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Role of Aurora kinase A in the regulation of apical-basolateral polarity during mouse epidermal development

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Doctoral thesis submitted to the Universidad Autónoma de Madrid for the degree of Doctor of Philosophy by M. Sci. in Molecular Biomedicine,

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CERTIFIES

That Ms Ljiljana Dukanovic, Master in Molecular Biomedicine by the Universidad Autónoma de Madrid, has completed her Doctoral Thesis **"Role of Aurora kinase A in the regulation of apical-basolateral polarity during mouse epidermal development"** and meets the necessary requirements to obtain the PhD Degree in Molecular Biosciences. To this purpose, she will defend her Doctoral Thesis at the Universidad Autónoma de Madrid. The Thesis has been carried out under my direction and hereby I authorize it to be defended to the appropriate Thesis Tribunal.

I hereby issue this certificate in Madrid on February 24th 2013.

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This thesis, submitted for the degree of Doctor of Philosophy at the Universidad Autónoma de Madrid, has been completed in the Epithelial Cell Biology Laboratory at the Spanish National Research Center (CNIO), under the supervision of

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Abstract

Epithelial cells are characterized by their polarized organization, which entails two structurally and functionally distinct domains: apical and basolateral. Interestingly, the proteins that participate in the establishment and maintenance of apical-basal polarity are also important players in the regulation of the orientation of asymmetric and symmetric cell divisions. Using mouse epidermis as a model system, we studied the mechanisms underlying the regulation of the polarized organization and oriented cell divisions of the basal progenitor cells. In particular, we explored the role of Aurora kinase A (AurkA), whose roles in cell polarity has been demonstrated in the *Drosophila* neuroblast, but still poorly understood in mammalian tissues. Using mouse genetics, we have observed that heterozygous inducible conditional knock-out mice in the epidermis present alterations in the polarized organization of epidermal cells short upon AurkA reductions. This is accompanied by hyperplasia and a wave of increase in the proliferation of basal epidermal cells. The localization of apical proteins Par3 and aPKC is also lost and the number of oriented of cell divisions parallel to the epidermal layer is aberrant. In addition, the formation of adherens junctions and tight junctions, which is closely related to the acquisition and maintenance of apical-basolateral polarity, is aberrant in the absence of AurkA. Additionally, we discovered that the localization of the active form of AurkA mirrors that of the Par complex proteins both *in vivo* and *in vitro*, suggesting a role for AurkA in the maintenance of cell polarity in the epidermis. Genetic ablation or chemical inhibition of AurkA leads to the mislocalization of the Par complex proteins *in vitro*. Knock down of AurkA in MDCK cells, a bona-fide model for studying apical-basal polarity, leads to the loss of aPKC from the cortex and malformation of 3-D cysts. Overall, our data suggest that AurkA also may play a role in the maintenance of apical-basolateral polarity in the mammalian epidermis, in addition to its canonical role in cell cycle, extending the implications of its function in the maintenance of tissue homeostasis.

Resumen

Las células epiteliales se caracterizan por presentar una organización subcelular polarizada, con dos dominios diferentes a nivel funcional y estructural: el dominio apical y el dominio basal. Resulta interesante observar que las proteínas implicadas en la formación y el mantenimiento de la polaridad apico-basal, desempeñan también un importante papel en la orientación de las divisiones celulares, de manera simétrica o asimétrica. En esta tesis hemos utilizado la piel de ratón como sistema modelo para estudiar los mecanismos que regulan la polaridad celular y la orientación de las divisiones de las células basales progenitoras. En concreto, hemos explorado el papel de la proteína Aurora Kinasa A (AurkA), la cual está implicada en la regulación de la polaridad de los neuroblastos de *Drosophila*, pero su contribución a la polaridad celular en tejidos de mamífero se desconoce. Utilizando modelos de ratón modificados genéticamente, hemos observado que ratones heterocigotos "knock-out" condicionales-inducibles en la epidermis para AurkA, presentan alteraciones en la polaridad de las células de la epidermis como consecuencia de la reducción de los niveles de AurkA. Además, la epidermis de estos ratones presenta hiperplasia y un aumento en la proliferación de las células basales progenitoras. La localización apical de las proteínas Par3 y aPKC se pierde y la orientación de las divisiones de las células basales está alterada. Las células de la epidermis de estos ratones presentan alteraciones en la formación de las uniones adherentes y las uniones estrechas, la cual está íntimamente relacionada con la formación y el mantenimiento de la polaridad apico-basal. Asimismo, hemos descubierto que la localización de la forma activa de AurkA coincide con la distribución de las proteínas del complejo Par, tanto *in vivo* como *in vitro*, lo cual sugiere que AurkA está implicada en el mantenimiento de la polaridad celular en la epidermis. Tanto la eliminación del gen como la inhibición química de la proteína AurkA conducen a la pérdida de la correcta localización de las proteínas del complejo Par *in vitro*. La disminución de los niveles de AurkA en las células MDCK (un modelo celular ampliamente utilizado para estudiar la polaridad apico-basal) induce una pérdida de la proteína aPKC de la corteza celular y la aparición de malformaciones en cultivos epiteliales en tres dimensiones (3D) *in vitro*.

De forma global, nuestros resultados indican que la proteína AurkA está implicada en el mantenimiento de la polaridad apico-basal en la epidermis de mamíferos. Esta nueva función de AurkA extiende las implicaciones de esta proteína en el mantenimiento de la homeostasis de los tejidos más allá de su papel canónico en la regulación del ciclo celular.

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Abbreviations

α-CAT	Alpha-catenin
β-CAT	Beta-catenin
ACD	Asymmetric cell division
AP	Adaptor Protein
AJ	Adherens Junctions
aPKC	Atypical Protein Kinase C
AurkA	Aurora Kinase A
BrdU	Bromodeoxyuridine
CRB	Crumbs complex
DAPI	4',6-diamidino-2-phenylindole
DLG	Disc Large
ECAD	E-cadherin
ECM	Extracellular Matrix
H&E	Hematoxylin and Eosin
IF	Immunofluorescence
IP	Intraperitoneal
K5	Keratin 5
K10	Keratin 10
K14	Keratin 14
KD	Knock down
LC	Low Calcium
LGL	Lethal giant larvae
MDCK	Madin-Darby Canine Kidney Epithelial Cells
MTOC	Microtubule Organizing Center
NC	Normal Calcium
OCT	Optimal cutting temperature compound
PAR	Partitioning Defective Protein
pAurkA	phospho AurkA
PATJ	PALS1-associated tight junction
PBS	Phosphate Buffered Saline
PCAD	P-Cadherin

PFA	Paraformaldehyde
PHH3	phospho histone H 3
SCD	Symmetric cell division
SJ	Septate junctions
TJ	Tight junctions
WB	Western blot

Introduction

1. Cell Polarity

1.1. What is cell polarity?

Cell polarity is a fundamental feature of almost all cell types. It refers to the existence of functionally and structurally distinct cellular compartments within cells, characterized by spatial differences of the cellular membrane, distinctive positioning of organelles, or the directionality of structural components, such as the intracellular cytoskeleton (Martin-Belmonte and Perez-Moreno, 2012). Cell polarity is essential during morphogenesis, and for the maintenance of the homeostasis of adult tissues. It regulates a wide range of physiological processes including directed cell migration during development and wound repair, the acquisition and maintenance of cell shapes and tissue organization, polarized transport and secretion of molecules across cell layers, and the orientation of the plane of cell division (Thompson, 2013).

The mechanisms that regulate cell polarity have been conserved throughout the eukaryotic kingdom. From unicellular cells such as yeast to multicellular organisms, including worms, flies and mammals, cell polarity proteins have been shown to exert parallel functions (Tepass, 2012; Macara and Mili, 2008). All these different models have since been utilized to study cell polarity and its involvement in the spatio-temporal regulation of diverse physiological events. A type of polarity that has been extensively studied in simple organisms is apical-basolateral polarity. This kind of cell polarity is characteristic of epithelial tissues, and it refers to the differential distribution of proteins and lipids into the apical and basolateral domains of the cell, and the positioning of organelles along the apical-basolateral axis. In epithelial tissues it is known to regulate morphogenesis, as well as maintenance of the tissue structure and integrity. However, our knowledge about the apical-basolateral polarity is only beginning to extend to the more complex, stratified epithelia.

1.2. Apical-basolateral polarity in simple epithelia

Apical-basolateral polarity in simple epithelia has been widely studied for many years in simple organisms such as *Drosophila melanogaster*, and it was not until the late 1970's that it started to be explored in mammalian cells (Laprise and Tepass, 2011). Cells of the simple epithelium, a one-cell thick epithelial tissue, are columnar and present remarkable apical-basolateral polarity features that allow the separation of the outer luminal space from internal compartments. Firstly, the apical surface of the epithelial cell's membrane, which faces the luminal space, displays specialized membrane structures, such as microvilli and cilia. These structures are particularly important for the secretion, absorption and excretion of molecules and solutes across epithelial layers (Muthuswamy and Xue, 2012). The basal membrane attaches to the extracellular matrix (ECM), associating epithelia to their adjacent connective tissues, whereas through the lateral membrane, neighboring cells are held together sustaining the maintenance of cohesive epithelial layers.

The spatiotemporal establishment of epithelial apical-basolateral polarity can be generally subdivided in four basic steps: 1) Epithelial cells receive an external signal arising from the ECM and/or neighboring cells, 2) A downstream signaling cascade of events involving the cell polarity machinery leads to the asymmetric compartmentalization of the cell membrane and organelles within the cell, 3) In parallel, cell polarity proteins are distributed to their appropriate domains; 4) Mitotic spindles orient along the established cell polarity axis (Figure 1)(Ahringer, 2003).

1.2.1. Initiation of polarization: contact as an external signal

The initial stages that lead to the establishment of apical-basolateral polarity involve the stimulation of epithelial cells by external cues. These signals are mainly mediated by the physical attachment of epithelial cells to the ECM, as well as the contact between neighboring cells. The initial contacts between cells lead in turn to the formation of cell-cell junctions. Two types of cell-cell junctions, adherens junctions (AJs) and tight junctions (TJs), have been shown to play a fundamental role in the establishment, as well as the maintenance of apical-basolateral polarity. Their formation initiates polarized rearrangements of the cytoskeleton, and they facilitate the proper assortment of apical

and basolateral proteins to their specific domains (Perez-Moreno and Belmonte, 2012). (Figure 2A).

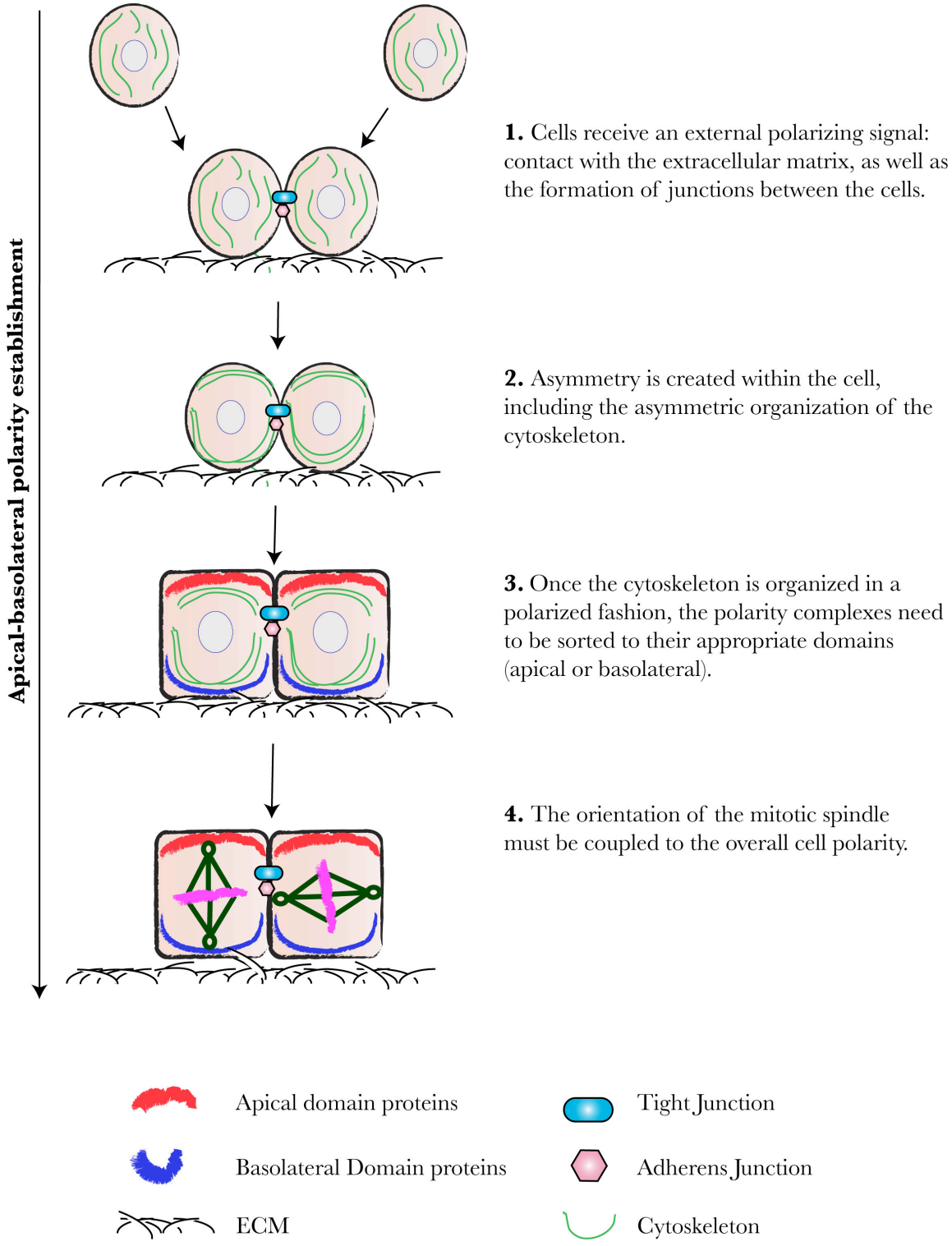


Figure 1 Establishment of apical-basolateral polarity in simple epithelia

Adherens junctions are essential for the development and physiology of epithelial tissues. In simple epithelia they are positioned below the TJs, and one of their main functions is to maintain the physical contact between epithelial cells (Meng and Takeichi, 2009) (Figure 2A). Two adhesive molecular units constitute AJs: the nectin-afadin complex and the classical cadherin-catenin complex (Niessen, 2007). The cadherin-catenin complex is composed of cadherin and catenin molecules (Harris and Peifer, 2004). Cadherins are calcium dependent proteins, thus the function and formation of the cadherin/complex strictly depends on the presence of extracellular calcium (Meng and Takeichi, 2009). During the initial stages of cellular interactions, both nectin/afadin and the calcium dependent cadherin/catenin complexes anchor to the actin cytoskeleton to strength the connection between neighboring cells. As more contacts are established, they extend the developing lateral membrane domain of epithelial cells, zipping the intercellular space between neighboring cells (Adams and Nelson, 1998; Vasioukhin and Fuchs, 2001). This process is closely linked to the distribution of proteins of the cell polarity machinery, which will be described in the next chapter.

Tight junctions (Septate junctions in flies) are structures that distribute in the border between the apical and the lateral membrane domains. At this cellular location, TJ dynamically seal the membrane restraining the free diffusion of membrane proteins and lipids between the apical and lateral membrane. In addition, TJs function as a gate, regulating the passage of solutes of molecules through the intercellular space, thus maintaining the barrier function of epithelial layers. Tight junctions consist of an assortment of transmembrane proteins including the Junctional Adhesion Molecule (JAM), Claudins, and Occludins. These proteins associate through their cytoplasmic domain with scaffolding proteins, such as the *zonula occludens* proteins ZO-1, ZO-2, and ZO-3 (Niessen, 2007). Interestingly, at the initial stages of cadherin contact formation, the scaffolding protein ZO-1 is found associated to α-catenin to later distribute to TJs. This allows the establishment of TJs, and the maintenance of apical-basolateral polarity. The hierarchy of events regulated by these junctional complexes is therefore initially modulated by AJs. Of note, alterations in the functional activity, or loss of expression of

cadherins disturb TJs. However, the absence of either ZO-1 or ZO-2 has no effect on AJ functionality (Tunggal et al., 2005; Umeda et al., 2006).

1.2.2. Establishment of asymmetry within the cell

At nascent contacts, one of the first responses that lead to the establishment of cell polarity is the dynamic asymmetric rearrangement of the microtubule and actin cytoskeletons. Microtubules are formed by tubulin dimers that polymerize forming microtubule bundles. These structures have fundamental functions including the positioning of organelles within the cell, the formation of polarized structures such as cilia, and mitotic spindles, in addition to serve as tracks for transporting molecules. Microtubules growth from the microtubule organizing center (MTOC), which is usually the centrosome, towards the cell cortex. In addition, non-centrosomal microtubules are found organized along the apical-basal axis. In the context of apical-basolateral polarity, the asymmetric rearrangement of microtubules is like a road building that is necessary for vesicle trafficking and the proper targeting of newly synthesized polarity proteins to specific domains. Interestingly, microtubule cytoskeletal rearrangements and vesicular trafficking are not only necessary for the establishment of cell polarity, but they also seem to be regulated by cell polarity mechanisms as well. There is some evidence, for example, that polarity proteins are required for endocytic trafficking (Balklava et al., 2007). Moreover, it has been recently demonstrated that cadherins are required for the proper positioning of the centrosome and nucleus within the cell (Dupin et al., 2009).

The actin cytoskeleton also plays a fundamental role in the establishment of apical-basal polarity. The initial contacts between cells are established by the dynamic contacts between filopodial extensions of neighboring cells (Adams and Nelson, 1998; Vasioukhin and Fuchs, 2001). At these membrane protrusions, the actin cytoskeleton is organized into actin cables. Once productive AJs linked to the actin cytoskeleton are established, the actin cytoskeleton branches and allows the formation of the actin cortical ring, via the spatiotemporal signaling mediated by small Rho GTPases (Rac1, Cdc42 and RhoA), and the activation of myosin-dependent mechanical forces (Mège et al., 2006). This in turn, extends the interphase of cell-adhesion and facilitates the assortment of cell polarity proteins to their developing specific domains (Tanos and Rodriguez-Boulan, 2008).

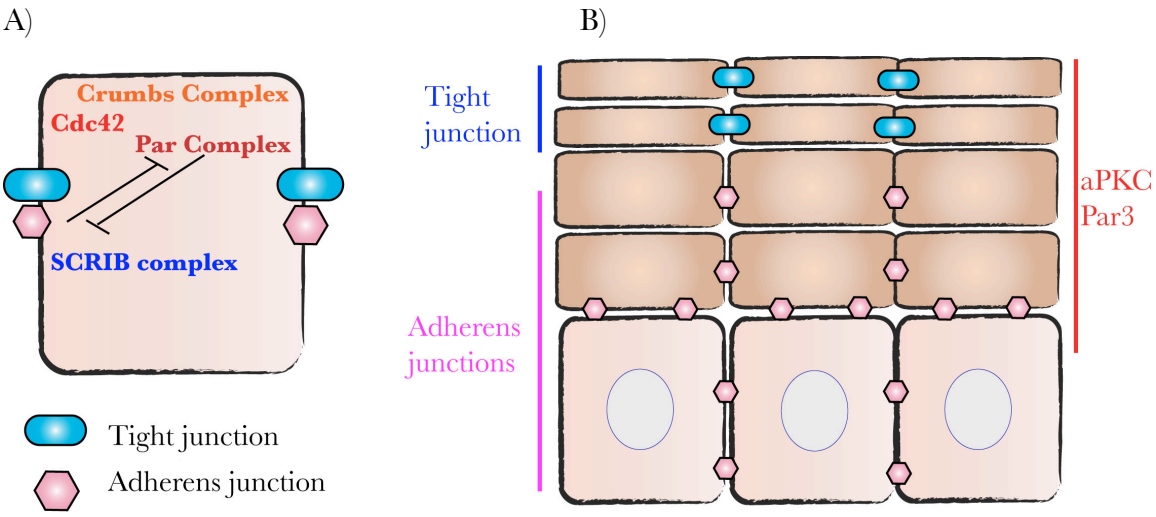


Figure 2. Schematic representation of the differences between apical-basolateral polarity in **A)** simple epithelia and **B)** skin epidermis.

1.2.3. Cell polarity complexes

Many polarity proteins have been identified so far (Table 1). At the core of the polarity machinery are three polarity complexes: the apically located 1) Par and 2) Crumbs (CRB) complexes, and 3) the basolaterally located Scribble (SCRB) complex.

Table1 Proteins of the cell polarity complexes

Drosophila melanogaster	**Mammals**
Bazooka (Baz)	PAR3
Par6 (Par6)	PAR6A to PAR6D
aPkc (aPkc)	PKC ι/λ and ζ
Crumbs (Crb)	CRB1 to CRB3
Stardust (Sdt)	PALS1
Patj	PATJ, MUPP1
Lethal giant larvae (Lgl)	LGL1, LGL2
Discs large (Dlg)	DLG1 to DLG3, SAP97
Scribble (Scrib)	SCRIB1

Modified from: Macara, 2004

The Par complex consists of the Partition defective 3 (Par3) and Partition defective 6 (Par6), which work together with atypical protein kinase C (aPKC) to regulate and maintain cell polarity (Chen and Zhang, 2013). The genes encoding for the three proteins were originally identified in genetic screens based in their requirement for the establishment of the anterior/posterior polarity of *C. elegans* zygotes. They were named 'Par' for the observed partitioning defectiveness of zygotes deficient in these genes (Kemphues et al., 1988). Par3 (Bazooka in *Drosophila*) and Par6 are scaffolding proteins and they are able to bind to each other as well as many other proteins, whereas aPKC is a kinase protein. Par6 binds aPKC and inhibits its kinase activity, but also serves to recruit aPKC substrates, including Par3 (Nagai-Tamai et al., 2002). The interaction of aPKC and Par3 plays an essential role in the polarization of the epithelial cells (Nagai-Tamai et al., 2002). Par3 initially localizes at nascent nectin/afadin adhesion complexes to later dissociate from the lateral domain to distribute to the apical domain to associate to the TJs protein JAM, upon activation of the small RhoGTPase Rac1 (Martin-Belmonte and Perez-Moreno, 2012). In the apical domain, another important protein that cooperates with the Par complex is the small RhoGTPase Cdc42. The recruitment of Cdc42-GTPase to the Par6/aPKC complex activates the kinase activity of aPKC (Joberty et al., 2000). These interactions ensure that the Par complex is localized properly to the apical domain. Interestingly, Par3 is able to bind with its N-terminal domain to microtubules indicating a close relationship between the cellular cytoskeleton and the polarity protein complexes (Chen and Zhang, 2013). It has also been demonstrated that the Par complex can regulate actomyosin contractions during *Drosophila* epithelial polarization (David et al., 2010)

The Crumbs complex, which consists of the transmembrane protein Crb and the proteins PALS1 and PALS1-associated tight junction (PATJ), is required for the establishment of the apical membrane. Par3 and Crb proteins are somewhat redundant. Studies have shown that in tissues lacking Crb, Par3 can compensate for its loss to allow the proper polarization of epithelial cells (Thompson et al., 2013).

The SCRIB complex consists of Dlg (Discs large), Scrib (Scribble) and Lgl (Lethal giant larvae), and they define the basolateral domain. Scribbled (Scrib) is a cadherin/catenin

junction-localized protein (Navarro C et al., 2005).

Polarity Complex interactions What do we know about the molecular journey of these proteins, upon polarization? The interaction of the cell with the extracellular matrix and neighboring cells leads to the recruitment of Par3 to primordial AJs as mentioned above. Further dissociation of Par3 from AJs is necessary to allow its redistribution to TJs, allowing the recruitment of TJs-associated proteins and the establishment of mature TJs. This dissociation requires the phosphorylation of Par3 by aPKC, and the two, together with Par6 redistribute to the apical domain.

At the same time, SCRIB promotes the basolateral membrane identity. The Par Complex has an antagonistic reaction with the basolateral proteins, including Par 1 and SCRIB complex proteins, which ensures that the proteins of the two domains do not mix. Some biochemical studies have provided some clues as to how apical proteins, through their antagonistic interactions, can be brought to the apical domain. For example, Par1 is known to phosphorylate the oligomerization domain, lipid binding-domain, and aPKC binding domain of Par3, driving Par3 to associate with the apical membrane by oligomerizing and interacting with aPKC (Izumi et al., 1998). However, it remains unclear how apical determinants restrict Par1 to the basolateral domain. Overexpression of aPKC or knock-down of Lgl enlarges the apical domain at the expense of lateral domain, whereas loss of aPKC activity or overexpression of Lgl reduces the apical domain (Humbert et al., 2003). Overall, these studies suggest that the interaction between the apical and basolateral proteins complexes at the cortex leads to mutual exclusion to sustain the identity of the plasma membrane domains.

Interestingly, studies in yeast have also shown that the polarized localization of these protein complexes is not only achieved through antagonistic interactions, but also through a simple positive-feedback loop in which the rate of Cdc42 recruitment to the bud site depends on the concentration of Cdc42 already residing at this location (Sardon et al., 2010). However, this phenomenon has not been yet demonstrated in mammalian tissues.

The intimate connection between polarity complexes to AJs and TJs highlights its functional codependence. Namely, it has been observed that the disruption of junctions and their components leads to loss of cell polarity. For example, in mouse keratinocytes,

loss of ECAD and PCAD leads to the loss of the polarized localization of aPKC and Par3 (Tinkle et al., 2008). Conversely, there is evidence that the disruption of polarity proteins can lead to problems in junction formation and/or stability, suggesting that apical-basolateral polarity is important for the cellular functions of these adhesion complexes, such as junction formation. It has been shown in the *Drosophila* epithelium, that the recruitment of ECAD into apical junctions requires Bazooka (Par3) (Harris and Peifer, 2004). Similarly, in the skin one study showed that Par3 controls the assembly of tight junctions through the Rac exchange factor Tiam1 (Chen and Macara, 2005). In summary, the complexity of cell polarity lies in the great number of interweaving interactions among all it's components, including junctions, cytoskeleton, and cell polarity complexes.

1.2.4. Spindle orientation

Natural world is spawned by incredible cell diversity. Through their development, multicellular organisms must give rise to different types of tissues, and this process implies that mother cells give rise to one daughter differentiated cell and another that maintains its progenitor characteristics. This asymmetric cell division is achieved through a precisely oriented cell division. This process is defined through two, mutually coupled events: 1) establishment of polarity, which implies unequal distribution of polarity proteins; and 2) alignment of the mitotic spindle with the respect to the polarity axis (McCaffrey and Macara, 2011). Thus, for asymmetric cell division to occur, cells have to rotate their mitotic spindles allowing the differential distribution of cell fate determinants between the mother and the differentiating cell. Oriented cell divisions are not only important during development, but also for the maintenance of the homeostasis of adult tissues. For example, in the brain during the division of neuroblasts, mitotic spindles must align with the established posterior/anterior polarity axis, ensuring the proper asymmetric cell division. Therefore, cell fate can be the result of cell polarity. The fundamental roles of asymmetric cell divisions in the specification of cell fate have already been determined in several mammalian tissues including the brain, the mammary epithelia, the lung, the intestine and the epidermis (Martin-Belmonte and Perez-Moreno, 2012). Genetic identification of the spindle orientation regulators occurred nearly two decades ago and the list of proteins required for proper spindle orientation has grown tremendously.

Among these regulators are the cell polarity complexes. For example, it was shown in *C. elegans* that loss of Par3 leads to aberrant spindle orientation, during the asymmetric division of the first embryonic cleavage (Cheng et al., 1995; Etemad-Moghadam et al., 1995). In mammals, loss of Par3 leads to loss of asymmetric cell divisions, leading to an increase in symmetric divisions and the expansion of tumor cells in the epidermis or mammary epithelia (McCaffrey and Macara, 2009; Niessen et al., 2012). In addition, the loss of tumor suppressor genes, such as p53, or APC has been correlated with the loss of oriented cell divisions and the proliferation of tumor cells in the mammary epithelia (Cicalese et al., 2009), or in the gut (Quyn AJ et al., 2010). Overall, these results highlight the relevance of studying the mechanisms that control epithelial cell polarity during development and disease.

2. The Skin

2.1 Organization of the skin

The largest organ of our body, the skin, has evolved to play a fundamental role in the maintenance of the body's homeostasis. It offers protection, sensation, thermoregulation, and absorption. While protecting the body, skin is exposed to many stresses, which can lead to damage and disease, such as skin cancers (Fuchs and Horsley, 2008).

The skin consists of: epidermis (an ectoderm-derived epithelial tissue), the underlying dermis (of mesodermal origin, composed of mesenchymal cells and ECM), subcutaneous fat, and the epithelial appendages, such as hair follicles and sebaceous glands (Fuchs, 2007). The epidermis, which forms the outermost layer of the skin, is a self-renewing, stratified epithelia that consist of several layers. The basal layer is the proliferative one, which gives rise to the upper, differentiated postmitotic layers of the epidermis via a fine-tuned terminal differentiation program: spinous (*Stratum spinosum*); granular *(Stratum granulosum)*, and the outermost, cornified layer (*Stratum corneum*) (Fuchs and Horsley, 2008). The maintenance of this epithelial structure in the skin is essential for tissue homeostasis,

and it is greatly dependent on functional cellular junctions and the polarized organization of the cells across the skin tissue as a whole.

2.2 Apical-basolateral polarity in the epidermis

The establishment and maintenance of apical-basolateral cell polarity in the epidermis is not only important for tissue architecture, but also for the regulation of oriented cell divisions, and the formation of a functional protective barrier. Like simple epithelia, the cells of the basal progenitor layer of the epidermis present intrinsic apical-basolateral polarity, and many polarity players found in simple epithelia are also found in the skin epithelium. However, when it comes to apical-basolateral polarity there are many differences between these two types of epithelia, since the stratified epidermis presents a tissue level of polarity, which makes polarity in tissues like skin more complicated to understand. First, unlike simple epithelia, where TJs are positioned in the border of the apical lateral boundaries of the two membrane domains within the cells, in stratified epithelia TJs are only present in the uppermost granular layer, also performing their specialized barrier functions (Niessen, 2007)(Fig 2B). Secondly, the Par complex proteins are expressed in the apical domain of basal progenitor cells, but distribute around the whole cell cortex of suprabasal-differentiated cells (Harris and Peifer, 2004)(Figure 2B). This unique design of polarity in stratified epithelia is still poorly understood, and further works is needed to understand the mechanisms involved in achieving such specialized level of polarization.

Further, there is some evidence indicating that in the skin epidermis, like in the simple epithelia, the proteins of the AJs and TJs can regulate the distribution and function of the polarity complexes (Harris and Peifer, 2004). Cadherin-deficient epidermis displays defective localization of Par3, aPKC, and Scribble. Conditional ablation of alpha-catenin (α-cat), a component of adherens junction, in the epidermis induces hyperplasia and loss of polarity (Vasioukhin and Fuchs, 2001; Lechler and Fuchs, 2005). However, it is particularly puzzling to consider this link since AJs of the basal layer of the epidermis, unlike in the simple epithelia, are unpolarized and distribute around the whole cell cortex.

Despite the differences between simple epithelial cells and the segregation of the polarity complexes and cell junctions across the apical axis of the whole epidermis, several functional parallels are found in the regulation and codependence of cell adhesion, and cell polarity events. There is some evidence that Par complex members can affect junctional stability in skin. It has been shown that aPKC activity is necessary for the formation of TJs in stratifying keratinocytes in vitro (Denning, 2007). In recent years both Par complexes and aPKC have also been implicated in the maintenance of the polarized architecture of skin and alterations in the expression of aPKC leads to barrier function defects (Denning, 2007; Niessen et al., 2012).

2.2.1 Apical-basolateral polarity and asymmetric cell division in skin

Besides the Par complex, Inscuteable and LGN are other two polarity proteins that are conserved, and in skin epithelia are found colocalizing apically with Par3, only during mitosis (Lechler and Fuchs, 2005). This Par3/Insc/LGN complex is very important for the recruitment of the effectors of spindle orientation-molecules that can interact with and generate force on the mitotic spindle. These effector proteins include NuMA and the cell motor complex formed by dynein/dynactin. NuMA is a microtubule binding protein that localized to both spindle poles and the apical cortex in asymmetrically dividing skin epithelial cells (Williams et al., 2011). Symmetric and asymmetric cell divisions (SCD and ACD) are key to the balanced proliferation and differentiation in the epidermis. Several studies have shown that apical-basolateral polarity, together with its complexes and junctions, can regulate the orientation of cell divisions, thus affecting ACD and SCD. Induction of INSC induces perpendicular cell division orientation, shortly after induction, and 3 days later, the effect is opposite (Poulson and Lechler, 2010). Knock down of LGN or NuMA leads to the bias towards parallel cell division (Williams et al., 2011). Interestingly, this process seems to regulate the Notch dependent differentiation of basal progenitor cells (Williams et al., 2011). It has been also shown that the loss of adhesive cues, such as integrin beta1 or α-cat in mouse epidermis results in random spindle orientations (Lechler and Fuchs, 2005).

3. Polarity and cancer

There is a growing amount of data showing that the localization or the expression of some of the polarity proteins is changed in many human cancers (McCaffrey et al., 2012). For example, Par complex proteins are often overexpressed in many human cancers (Table 2). However, our understanding of the role of polarity in cancer in mammals is still poor. It has been suggested that aPKC could be an oncoprotein, since its expression is usually elevated in human epithelial cancers, including lung, liver, pancreas, ovarian, prostate, and colon (Martin-Belmonte and Perez-Moreno, 2012). One study showed that aPKC functions downstream of RAS signaling and upstream of Rac1GTPase, in lung and colon carcinogenesis. LKB1 (Par4 in *Drosophila*), is a serine/threonine kinase that also regulates cell growth and polarity in metazoans, whose mutation has been also directly associated with human cancers (Jansen et al., 2009). Epidermal inactivation of LKB1 predisposes mice to non-melanoma skin cancer, thus providing direct evidence that LKB1 serves as a cell-autonomous tumor suppressor in the skin (Gurumurthy et al., 2008). Interestingly, LKB is also linked to metabolic pathways and AMPK signaling.

SCRIB has also been implicated in playing a tumor suppressor role. It has been demonstrated recently, *in vivo* and *in vitro*, that loss of SCRIB decreases apoptosis and increases cellular trasformation via the MYC signaling pathway (Wu et al., 2012).

Another study has demonstrated that silencing of Par3 in the mammary glands reduced the latency of tumors expressing oncogenic Notch or Ras (Cafely et al., 2012). Par3 depletion was associated with induction of MMP9, destruction of the extracellular matrix, and invasion. This was mediated by aPKC-dependent JAK/Stat3 activation. This is important because Par3 is significantly reduced in human breast cancers, which correlates with active aPKC. Ablation of Par3 in the epidermis inhibits formation and growth of papillomas. However, those mice are predisposed to the formation of keratocanthomas (Iden et al., 2012).

Gene (protein)	Alterations	Cancer type	Phenotypes
Crumbs complex			
CRB3 (crumbs 3)	Downregulated expression	Human tumour epithelial-derived cell lines	Cell–cell junctions disrupted and increased metastasis
PAR complex			
PARD3 (PAR3)	Gene deleted or downregulated expression	Oesophageal squamous cell carcinoma cell lines and primary tumour tissue	Cell–cell junctions disrupted
PARD6A (PAR6α)	Overexpressed	ER-positive breast cancer cell lines and primary tumour tissue	Hyperproliferation
	Overexpressed and phosphorylated	Human BRCA1-defective tumour tissues	Lumen filling, cell–cell junctions disrupted and increased metastasis
	Overexpressed	Stromal cells in non-small-cell lung cancer tissue samples	Associated with good prognosis
PRKCZ (PKCζ or aPKC)	Overexpressed	Human hepatocellular carcinoma samples	Hyperproliferation
	Overexpressed	Bladder tumour cell lines and primary tumour tissues	Correlated with invasiveness
	Overexpressed and phosphorylated	Dysplastic oral epithelial tissue samples, squamous cell carcinoma of the head and neck tissue samples and cell lines	Increased cell proliferation
	Overexpressed	Pancreatic cancer tissues	Invasive and metastatic phenotype
	Overexpressed	Samples of hyperplastic enlarged lobular units of precancerous breast lesion	Increased cell proliferation
PRKCI (PKCι or aPKC)	Gene amplified and protein overexpressed and mislocalized	Ovarian cancer tissue samples	Associated with low survival rate
	Overexpressed and phosphorylated	Hepatocellular carcinoma tissue samples	Associated with metastasis and invasion
	Overexpressed	Non-small-cell lung cancer cell lines and primary tumour tissues	Associated with poor prognosis
	Overexpressed	Primary breast cancer tissue samples	Associated with larger tumours, invasion and metastasis
	Overexpressed	Pancreatic cancer tissue samples	Tumour angiogenesis and metastasis

Table 2. Alterations of PAR complex proteins in human cancers (modified from Martin Belmonte and Perez-Moreno, 2012)

A way in which defects in apical-basolateral proteins can act as a tumor initiation is by deregulating spindle orientation, and thus the balance between symmetric and asymmetric cell division. Prior studies in *Drosophila* have shown that switching from ACD to SCD promotes unrestricted growth and tumor formation (Januschke and Gonzalez, 2008). In the context of cancer initiation, alterations in spindle orientation and asymmetric cell division have been implicated in the acquisition of tumorigenic characteristics (Quyn et al., 2010). Using lineage-tracing approaches this study demonstrate a clear correlation between spindle orientation and the occurrence of asymmetric cell divisions. Furthermore, this correlated with an increase in perpendicular spindle orientations and reductions in ACDs in the pre-cancerogenic gut of the APC deficient mouse model (Quyn et al., 2010). Similarly, the loss of the tumor suppressor

gene p53 has also been associated with an increase in SCD of mammary carcinoma cells (Cicalese et al., 2009). As another example, the Lgl knock-out (KO) mice present randomized cell divisions in neural progenitors, causing severe brain dysplasia (Klezovitch et al., 2004).

One of the most direct evidences making the link between cancer and polarity is provided in the study showing that the TGFβ receptor directly interacts with Par6 and phosphorylates Par6 (Ozdamar et al., 2005). This phosphorylation event is necessary for the TGFβ-dependent Epithelial to Mesenchymal Transition (EMT) in mammary gland epithelial cells. However, our understanding of the molecular links between cell polarity proteins and cancer initiation and progression is still very poor.

4. Aurora kinase A

1.1 A mitotic protein that functions beyond mitosis

Aurora Kinase A (AurkA) is also known as serine/threonine kinase 15 (STK15), serine/threonine kinase 6 (STK6), breast tumor amplified kinase (BTAK), and Aur-related kinase 1 (ARK1). As the names indicate, AurkA is a serine/threonine kinase that is part of the Aurora /IPL1-related kinase family, that comprises of AurkA, AurkB and AurkC. It's a 403- amino acid protein with a canonical kinase domain and it is mostly known for its role during mitosis, although new non-mitotic roles of the protein are now emerging, including the control of cell polarity (Nikonova et al., 2013).

4.1.1. Mitotic role of AurkA

During mitosis AurkA plays a role in centrosome maturation, timing of mitotic entry, and the construction and control of bipolar spindle. It begins to accumulate significantly at the centrosomes in the S phase and is activated at the transition between the G2 and M phases. This accumulation of AurkA at the centrosome allows for the nucleation of the microtubules that form the mitotic spindle. In this way AurkA is necessary for the trigger of the mitotic entry. Once activated, AurkA moves down the spindle to the midzone of

the plane of division, after which it facilitates chromatin separation and cytokinesis (Fu et al., 2007).

In a recent computational study integrating different sources of biological information, a list of 90 AurkA substrates was generated (Sardon et al., 2010). Some of these substrates activate AurkA and others are activated by AurkA. The activation and degradation of AurkA is regulated spatio-temporally by many proteins. One of the major events is the regulation of AurkA via phosphorylation. This can be triggered by several proteins, which in turn can then lead to the auto-phosphorylation of AurkA. The most studied AurkA regulatory proteins are TPX2, Ajuba, NEDD9 and BORA (Vader and Lens, 2008). At the breakdown of the nuclear envelope, TPX2 is released from an inhibitory complex and binds to AurkA. This binding leads to a conformational change of AurkA that allows the access for AurkA substrates. This interaction also helps AurkA to be targeted to the mitotic spindle. Regarding other interactions, it has been shown that the association of AurkA with Ajuba is important for the activation of cyclinB/CDK1 complex and the mitotic commitment (Vader and Lens, 2008). Other substrates of AurkA include BRACA-1, p53, Eg5, CDC25B (Kollareddy et al, 2008).

4.1.2. Non-mitotic roles of AurkA

Recently, several lines of evidence have emerged suggesting that AurkA functions beyond its fundamental role in mitosis. Particularly, there are studies showing that AurkA is involved in microtubule dynamics, cell migration, disassembly of cilia, and cell polarity. Chemical inhibition of AurkA, in mammalian cells in interphase, leads to the abrogation of microtubule dynamics (Lorenzo et al., 2009), suggesting that a pool of AurkA is active in non-centrosomal compartments. It has also been demonstrated that AurkA plays a role in neurite elongation and migration where the interaction between NEDD9 and AurkA is responsible for the effect (Mori et al., 2009). In the unicellular organism *Chlamydomonas* it has been shown that the AurkA ortholog CALK regulate flagellar length (Pugacheva et al., 2007). Interestingly, in a parallel way in mammalian cells, AurkA via interaction with NEDD9, function in the basal body of the cilium in non-cycling G0/G1 cells to promote ciliary reabsobtion (Pugacheva et al., 2007).

4.1.3. The emerging link between AurkA and polarity

In 2002, Berdnik and Knoblich showed that AurkA is required for centrosome maturation and actin-dependent asymmetric protein localization. In 2008, Wirtz-Peitz et. al demonstrated that in the *Drosophila* neuroblast, AurkA phosphorylates the Par complex linking cell polarity with asymmetric cell division. In this pathway, AurkA plays a role in phosphorylating Par6, which leads to the activation of aPKC and replacement of Lgl by the Bazooka protein, within the Par complex. This is required for the release and exclusion of Lgl from the cortex of one of the dividing cells to the other, in addition to the proper localization of the protein NuMa. Another evidence that has linked AurkA with cell polarity is the identification of the existence of aPKC-AurkA-NDEL1 pathway, which is crucial for the regulation of microtubule organization during neurite extension (Mori et al., 2009).

There are several studies describing AurkA's involvement in symmetric and asymmetric cell division. A very recent work shows that AurkA regulates mammary epithelial cell fate by regulating spindle orientation in a Notch-dependent manner (Regan et al., 2013). Another study showed that the complete deficiency of AurkA in epidermal cells during skin development leads to alterations in cell division, and thus stratification (Torchia et al. 2013). Although this was mainly attributed to the mitotic roles of AurkA, stratification, although aberrant, still occurred suggesting a possible involvement of AurkA in the balance of symmetric and asymmetric cell divisions of epidermal progenitor cells.

4.2. AurkA and cancer

Many solid tumors, including colorectal, breast, ovarian, prostate, neuroblastoma, and cervical cancers present an increase in AurkA levels. AurkA is situated on chromosome 20q13.2, a locus that is frequently amplified in solid tumors (Vader and Lens, 2008). However, in some cases AurkA is increased in the absence of the chromosomal rearrangements. Overexpression of AurkA transforms NIH3T3 cells, and when these cells are injected into nude mice, they grow tumors (Fu et al., 2007). AurkA is transcriptionally regulated by several transcription factors, including STAT5a, estrogen/GATA3, HIF1.

In cancers, some of these transcription factors are misregulated, leading to the aberrant levels of AurkA (Ochi et al., 2009). The result of AurkA overexpression is the presence of multiple centrosomes, which leads to multiple spindles, increased resistance to apoptosis and deficient cell cycle checkpoint. Interestingly, however, the overexpression of AurkA is not sufficient to induce tumorigenesis. But in a p53 deficient background, in a mouse model of mammary carcinogenesis, the overexpression of AurkA was able to induce tumorigenesis (Wu et al., 2012). Normally, AurkA overexpression leads to the spindle check-point activation or clearance of cells by apoptosis. However, loss of p53 eliminates this check-point, leading to aberrant cell divisions and genomic instability. Thus the proposed model is that AurkA overexpression causes centrosomal amplification, which would normally lead to the check-point activation and apoptosis. However, when cells lose p53, they are overriding checkpoints and continuing with the cell division, which can lead to aneuploidy and cancer (Wu et al., 2012).

For these reasons, AurkA has attracted a lot of attention as a potential therapeutic target. The scientific literature tends to consider elevated levels of AurkA as a predictive value of aggressiveness of cancers, although there is literature that contradicts this dogma. As of 2012 there are 9 compounds that target AurkA that have made it through preclinical testing into phase I and II (Ochi et al., 2009). The most promising one so far has been MLN8237 (alisertib), which has shown to have some effect in combination with other drugs .

Interestingly, in 2008 a study made by Lu et al. was published demonstrating that the AurkA$^{-/+}$genetic mouse model develops spontaneous tumors. Indeed the heterozygous mice presented 3-fold higher incidence of tumors. Tumors found were in various organs, including thymus, lung, liver and lymph node. Most tumors were lymphoma that invaded the lung.

It is understood that a way in which AurkA contributes to the tumor development is through genomic instability. However, lately, new roles or AurkA are being discovered, suggesting that there may be additional pathways through which AurkA may plays a role in cancer.

Objectives

Apical-basolateral polarity in epithelial tissues is fundamental during morphogenesis for the acquisition and maintenance of proper tissue architecture, polarized cell movements and oriented cell divisions. These important roles are also sustained in adult tissues preserving their physiological balance. Alterations in the functional activity of the proteins that partake in the cell polarity machinery have been associated with disease, including the acquisition of tumorigenic characteristics. Despite the relevance of these events, little is known about the mechanisms that regulate cell polarity in stratified epithelia, such as the skin. Using mouse genetic models and cell culture studies, the main goal of this thesis was to characterize the involvement of Aurora kinase A, a mitotic protein, in the regulation of the establishment and maintenance of apical-basolateral polarity of epidermal cells.

1. Characterize the epidermis of the K14CreERT2; AurkA$^{\Delta ep/+}$ and K14CreERT2; AurkA$^{\Delta ep/\Delta ep}$ mice during skin development.
2. Investigate the involvement of AurkA in the regulation of the acquisition and maintenance of the apical-basolateral polarity properties of epidermal cells, both *in vivo* and *in vitro*.

Objetivos

En los tejidos epiteliales la polaridad apico-basal es fundamental durante el desarrollo embrionario para la adquisición y el mantenimiento de una arquitectura tisular correcta, el movimiento polarizado de células y la orientación de las divisiones celulares. Estas funciones tan cruciales también preservan el balance fisiológico de los tejidos adultos. Las alteraciones en la actividad funcional de las proteínas que regulan la polaridad celular están asociadas con diversas enfermedades, entre ellas la adquisición de características tumorales. A pesar de la relevancia de estas proteínas, no se conocen bien los mecanismos que regulan la polaridad celular en tejidos epiteliales estratificados, como la epidermis de la piel. Utilizando modelos de ratón modificados genéticamente y cultivos celulares, el objetivo principal de esta tesis es caracterizar el papel de Aurora Kinasa A, una proteína del ciclo celular, en la regulación de la formación y el mantenimiento de la polaridad apico-basal en células de la epidermis.

1. Caracterizar la epidermis de ratones K14CreERT2; AurkA$^{\Delta ep/+}$ y K14CreERT2; AurkA$^{\Delta ep/\Delta ep}$ durante el desarrollo embrionario.
2. Investigar el papel de AurkA en la regulación de la formación y el mantenimiento de la polaridad apico-basal en células de la epidermis, *in vivo* e *in vitro.*

Methods

1. Mice

- **Mouse strains, mouse treatments, and embryo collection**

All the animal experiments performed in this study, were previously approved by the Animal Experimental Ethics Committee of the Instituto de Salud Carlos III (Madrid, Spain). K14CreERT2; AurkA $^{lox/lox}$ mice were kindly provided by the laboratory of Malumbres M. at CNIO. All matings were set at night and the presence of vaginal plugs was corroborated the following morning, which indicated day E0.5 of pregnancy. Starting on the E11.5 day of development, the *in vivo* recombination of the floxed *AurkA* gene was induced by the intraperitoneal administration of 3mg of tamoxifen (Sigma, St. Louis), dissolved in 100 μl of sunflower oil. Daily injections were maintained until the day of the collection of the embryos (E14.5, E15.5, E16.5, E17.5, E18.5). Alternatively, in a different set of experiments, two daily injections were given prior to the collection of the embryos. For the ablation of *AurkA* in adult mice, 5 daily injections of the same dosage of tamoxifen were administered intraperitoneally to mice before their sacrifice.

For the AurkA ablation in the adult mice, 5 daily tamoxifen injections of 3mg were sufficient.

The presence of heterozygous, homozygous floxed alleles, or deleted alleles was detected by PCR using the following primers:

- Primers that amplify the deletion allele (274 bp product)

Fw CAGAGTCTAAGTCGAGATATCACCTGAGGGTTGA

Rw GATGGAAACCCTGAGCACCTGTGAAAC

- Primers to screen for the presence of 2 products: 467 bp = wildtype, 594 bp = floxed allele

Fw CCTGTGAGTTGGAAAGGGACATGGCTG

Rw CCACCACGAAGGCAGTGTTCAATCCTAAA

K14CreERT2; AurkA$^{lox/lox}$ mice were crossed to Rosa26LacZ mice, in order to generate a reporter mouse model for the activity of Cre, upon tamoxifen administration.

For the performance of bromodeoxyuridine (BrdU) staining to monitor cell proliferation in vivo, pregnant females were injected with 2 mg of BrdU (Sigma, St. Louis), dissolved in 100μl of PBS, 3 h before the embryo collection.

- **Epidermal Permeability Barrier (EPB)**

Unfixed, untreated, freshly isolated embryos were rinsed in PBS. They were then submerged into 70% ethanol containing 0.01 mg of bromophenol-blue. They were kept for one hour at 37 °C, then rinsed well and analyzed.

- **Histology and Immunostaining**

For BrdU, X-gal, and Immunofluorescence (IF) stainings, unfixed embryos were embedded in optimal cutting temperature compound (OCT) and kept at -80C. Frozen embryos were then sectioned in the cryostat to obtain 7 μm sagital sections. For IF analyses, sections were first fixed with 4% paraformaldehyde for 10 min at room temperature (RT), washed with 1X PBS for 5 min, then blocked with the blocking buffer (0.3% Triton, 1% BSA, 1 % Gelatin, 5% normal goat serum, 5% normal donkey serum in 1X PBS) for 30 min. The incubation of primary antibodies was performed following the manufacturer's instructions.

For X-gal stainings, sections were fixed with the fixative solution (0.2% glutaraldehyde, 5 mM EGTA pH 8.0 in water), 2 mM MgCl2 and 0.1 M Sodium phosphate buffer pH 7.2) for 5-10 min at RT, then washed twice for 20-30 min at room temperature with a washing solution (0.1M Sodium phosphate buffer pH 7.2, 2 mM MgCl2, 0.1% Sodium deoxycholate, 0.02% Nonidet P40 in water). Slides were then transferred to a pre-warmed staining solution containing 1mg/ml X-gal (Promega, Fitchburg), 5mM K3Fe(CN)6, 5mM K4Fe(CN)6-3 H2O in washing solution. The slides were left at 37°C overnight, then washed three times with the washing solution, with mild agitation, at room temperature, and then four times with PBS. Slides were then stained with DAPI, dried and mounted.

For BrdU stainings, after fixation in 4% PFA in PBS, sections were washed well in PBS and incubated in 1N HCl at 37°C for 30 min. Then, the slides were blocked, and

incubated with primary and secondary antibody according to the manufacturer's instructions.

For Hematoxylin & Eosin (H&E) stainings and AurkA immunohistochemical analyses, embryos were fixed in formalin and embedded in paraffin. A standard protocol of deparafinization was followed and the sections were stained with AurkA antibodies following the protocol described above. For H&E stainings, after fixation in PFA, slides were incubated consecutively with hematoxylin and eosin for 5 min, with 3 PBS washes in between. Slides were dried and mounted for all the analyses.

2. Culture of mouse primary keratinocytes and cell treatments

- **Isolation and culture of primary keratinocytes**

Primary keratinocytes were isolated from the tail of adult K14CreERT2; AurkA $^{lox/lox}$ or K14CreERT2; AurkA $^{lox/+}$ mice. In brief, tail skin was incubated in dispase (Sigma, St. Louis) and trypsin (Gibco, Aucklan). After filtering cell suspensions through 40 μm cell strainers, cells were cultured in Low Calcium Medium – LC (0.05mM < Ca^{++}). They were cultured on a plate of Ca^{++}-free growing fibroblasts pretreated with mitomycin C (feeder cells). They were passaged twice onto plates with mitomycin C-treated feeders, until they became feeder-independent. Double E-cadherin KO; P-cadherin shRNA cells, as well as control cells, were kindly provided by E. Fuchs, Rockefeller University, NY.

- **Treatments and transfections of primary keratinocytes**

To ablate AurkA, 70% confluent cells were treated with 600 nM 4-OH Tamoxifen (Sigma, St. Louis) for 24 h. The absence of AurkA was tested by immunoblot and PCR. For the inhibition of phospho AurkA, the MLN8237-Alisertib inhibitor (Selleckchem, Houston) was administered to the cells, at a concentration of 15 μM. Almost 100 % of cells exhibited the loss of the pAurkA IF signal after 24 h incubation. Due to the reversibility of the binding of the molecule, the treatment with the inhibitor was

performed again after 24 h. The PKC ζ Pseudosubstrate (Millipore, Darmstardt) was used at a final concentration of 1 μM. Cells were analyzed 12 h after the treatment.

Primary mouse keratinocytes were transfected with a plasmid expressing AurkA-GFP (kindly provided by the laboratory of M. Malumbres, CNIO, Madrid), using the X-tremeGENE DNA Transfection reagent (Roche, Mannheim) according to the manufacturer's instructions.

- **Cytoochip and migration assay**

24 h after addition of tamoxifen in culture, cells were trypsinized and plated onto the umbrella-shaped, fibronectin coated commercial micropatterns (CYTOO, Grenoble), using the protocol provided by the manufacturer, with slight modifications. In brief, cytoochips were placed in a well of a 6-well plate. 60,000 cells were seeded per cytoochip and 4 ml of LC media were added to each well. Cells were allowed to attach to the Cytoochips for 9 h, the coverslips were gently washed with 1 X PBS, fixed and immunostained with an antibody that recognizes the Golgi marker (GM130), and DAPI, according to the protocol described above. For migration assays, cells were plated on coverslips, once attached, the *in vitro* recombination of AurkA was performed by the addition of tamoxifen. 24 h later, two small scratches were made with a small pipette tip on the confluent monolayer of cells. 9 h later, cells were fixed and stained for GM130 and DAPI.

- **Calcium switch assay**

Keratinocytes were grown to full confluency in LC medium, and then their media was replaced for a media containing 1.8 mM Ca^{++} (Normal Calcium media – NC), to induce the formation of cell junctions. Cells were washed with PBS, fixed and stained as indicated above at different time points to perform kinetic analyses of the formation of cell-cell junctions.

- **Immunofluorescence**

Primary mouse keratinocytes plated on coverslips were fixed with 4% PFA for 5 min, washed with PBS and blocked using the blocking solution (described above). Cells were incubated with primary antibodies according to the manufacturer's instructions, followed by incubation with fluorescence conjugated secondary antibodies.

- **Immunoblotting**

For analysis of total protein levels by immunoblotting, cells were lysed in RIPA buffer containing: 50 mM Tris-HCl pH 7.4, 1% NP-40, 0.2% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA and 0.2% SDS and proteinase inhibitors. Proteins were subjected to SDS-PAGE electrophoresis under reduced conditions, and transferred to nitrocellulose membranes. Primary antibodies were used to perform the immunoblot analyses according to the manufacturers' instructions. Immunoblots were developed using the ECL system (Invitrogen).

3. MDCK culture and treatments

- **MDCK culture**

MDCK II cells, kindly provided by the laboratory of Martin-Belmonte F. at CBM, Madrid, were cultured in MEM media (Life Technologies, Carlsbad), containing GlutaMAX (Gibco invitrogen, Carlbad), 5 % FBS and Pen/Strep.

- **MDCK treatments, electroporation and transfection**

The AurkA inhibitor MLN8237-Alisertib (Selleckchem, Houston) described previously was also administered to MDCK cells, at a concentration of 20 μM.

For the AurkA knockdown experiments, MDCK cells were electroporated using the NEON transfection system (Invitrogen, Carlbad), or the X-tremeGENE transfection reagent according to the manufacturer's protocol. A cocktail of two AurkA siRNAs with the following sequences was used: AurkA1: 5'-AUGCCCUGUCUUACUGUCA-3' ;

AurkA2: 5'-AACGUGUUCUGGUGACUCA-3' (Life Technologies, Carlsbad). The ON-TARGETplus Non-targeting siRNA (Thermo Scientific, Waltham) was used as a negative control. One million cells were transfected with 1 μg of each siRNA. 48 h after transfection, the reduction on the expression of AurkA was confirmed by IF analysis and immunoblot.

- **MDCK cyst formation on Matrigel**

Half a million cells were plated in one well of a 6-well plate. A day after, cells were washed with PBS for 15 min, trypsinized with 1 ml of trypsin and later neutralized with 1 ml of media, without centrifugation. In parallel, 8-well chamber slides were covered with 8 μl of Matrigel (BD, Franklin Lakes) and dried for 10 min. 2,500 cells were then seeded per well and grew in 250 μl of media containing 2 % Matrigel. 4 days later, the specific AurkA inhibitor was added to the media. After 20 h of incubation, cells were fixed and immunostained using 0.1% SDS in PBS to permeabilize the cells and incubated for 3 h with primary antibodies.

- **MDCK Calcium switch**

MDCK cells were grown in MEM media until confluency. Cells were washed 3 times in PBS for 5 min. Then MEM media without Calcium, supplemented with 5 % of Ca^{++}-chelated FBS was added to cells to promote the loss of cell-cell adhesion. 20 h later this medium was replaced by standard MEM media containing Ca^{++} to induce the formation of cell-cell contacts.

- **Immunofluorescence**

MDCK cells plated on the coverslips were fixed with -20 ºC methanol for 2 min, washed with PBS and blocked using the blocking solution described above. MDCK cells were incubated with primary antibodies according to the manufacturer's instructions, followed by incubation with fluorescencent conjugated secondary antibodies. Images were acquired using a Nikon fluorescent microscope, using the oil immersion objectives 40 X or 63 X.

4. Antibodies

PKC ζ rabbit antibody (sc-216, clone C-20, Santa Cruz), 1:500, pAurkA rabbit antibody (AB83968, Abcam, Cambridge), 1:500 dilution; AurkA mouse monoclonal antibody for IF (AB13824, Abcam, Cambridge), 1:300 dilution; AurkA mouse antibody for IHC (610938, BD Franklin Lakes); BrdU rat antibody (6326, Abcam, Cambridge), 1:1000 dilution; β-catenin mouse antibody (C7207, Sigma, St. Louis), 1:500; E-cadherin rat antibody (12-1900, Invitrogen, Carlsbad); Filaggrin rabbit antibody (PRB-417B, ATOM, Barcelona), 1:200 dilution; GM130 mouse (610822, BD, Franklin Lakes) 1:500; pHistoneH3 (06-570 Millipore, Billerica), 1:300; Keratin 5 chicken antibody (SIG-3475, Covance, Princeton), 1:500 dilution; Keratin 10 rabbit antibody (PRB-159P, Covance, Princeton), 1:300 dilution; Par3 rabbit antibody (07-330, Millipore, Billerica), 1:500 dilution; ZO1 and Occludin, (kindly provided by Pedro L. Majano, Hospital Universitario de la Princesa, Madrid) , 1:500.

Results

1. Characterize the epidermis of the K14CreERT2; AurkA$^{\Delta ep/\Delta ep}$ and K14CreERT2; AurkA$^{\Delta ep/+}$ mice during skin development

It has been recently documented that the complete loss of AurkA during epidermal development results in cell growth arrest and genomic instability (Torchia et al., 2013), hindering the probability to explore other potential functions of AurkA in epidermis.
In this project, by partially decreasing the expression of AurkA in epidermal development, we aimed to explore new AurkA facets at early stages of epidermal differentiation, and compare them to control mice with a homozygous loss of AurkA as well as controls.
In order to uncouple the defects arising from genomic instability from those related to other roles of AurkA, we decided to investigate the effect of the loss of AurkA shortly after the specific ablation of AurkA in the epidermis, using the K14CreERT2 inducible mouse system, both in AurkA homozygous (K14CreERT2; AurkA$^{\Delta ep/\Delta ep}$) and heterozygous (K14CreERT2; AurkA$^{\Delta ep/+}$) scenario. This provided us with a powerful system to explore the initial consequences of the partial loss of AurkA during skin development.

1.1. Kinetics of the genetic recombination event in K14CreERT2; AurkA$^{\Delta ep/\Delta ep}$ and K14CreERT2; AurkA$^{\Delta ep/+}$ mice upon Tamoxifen administration during skin development

The K14-CreERT2; AurkA$^{lox/lox}$ mice were previously generated (Pérez de Castro, 2013) using the AurkA^{lox} conditional allele (Cowley et. al, 2009) and the K14-CreERT2 transgene (Metzger et. al, 2005). This model allows the conditional ablation of AurkA in Keratin 14 (K14)-expressing tissues (including skin epidermis), upon tamoxifen administration. Since our objective was to test whether the loss of AurkA affects the apical-basolateral polarity during skin morphogenesis, we first validated the occurrence of

the gene recombination events at different time points of epidermal development before birth. The stratification of the epidermis occurs at E14.5-E15.5. For this reason, pregnant females were administered daily intraperitoneal (IP) injections of tamoxifen, starting from the E11.5 (day post coitum). Embryos were collected from E14.5 to the latest developmental E18.5 stage (Figure 3A). PCR analysis of the embryo DNA confirmed the presence of the lox alleles, as well as the AurkA deletion after tamoxifen injections (Figure 3B). Although the characterization of the K14CreERT2; AurkA$^{lox/lox}$ model in the adult mouse tissues has been published (Perez de Castro et al, 2013), we needed to characterize the model in the embryos. To further determine the timing of the Cre recombination event, K14CreERT2; AurkA$^{lox/lox}$ mice were crossed with the Rosa26LacZ reporter mice. Pregnant females were injected with tamoxifen, as indicated above, and the embryos were collected at E13.5, E14.5, E15.5 and E16.5. Then, the activity of ß-galactosidase was analyzed in frozen embryo sections, resulting in the appearance of the blue color, which indicates the occurrence of the Cre recombination event, which in our system started at E14.5 (Figure 3C).

1.2. Levels of AurkA in AurkA$^{\Delta ep/\Delta ep}$ and AurkA$^{\Delta ep/+}$ epidermis during development

To further analyze the earliest developmental stage in which the levels of AurkA were reduced in our system, we performed immunohistochemical analyses of AurkA in the skin of mouse embryos collected at different developmental time points (Figure 4A). AurkA positive cells in the epidermis were counted and quantified for three time points: E14.5, E15.5, and E16.5 (Figure 4B). These results showed that there was a significant decrease of the levels of AurkA in the epidermis of both heterozygous and homozygous embryos, compared to their control counterparts, which started from E14.5.

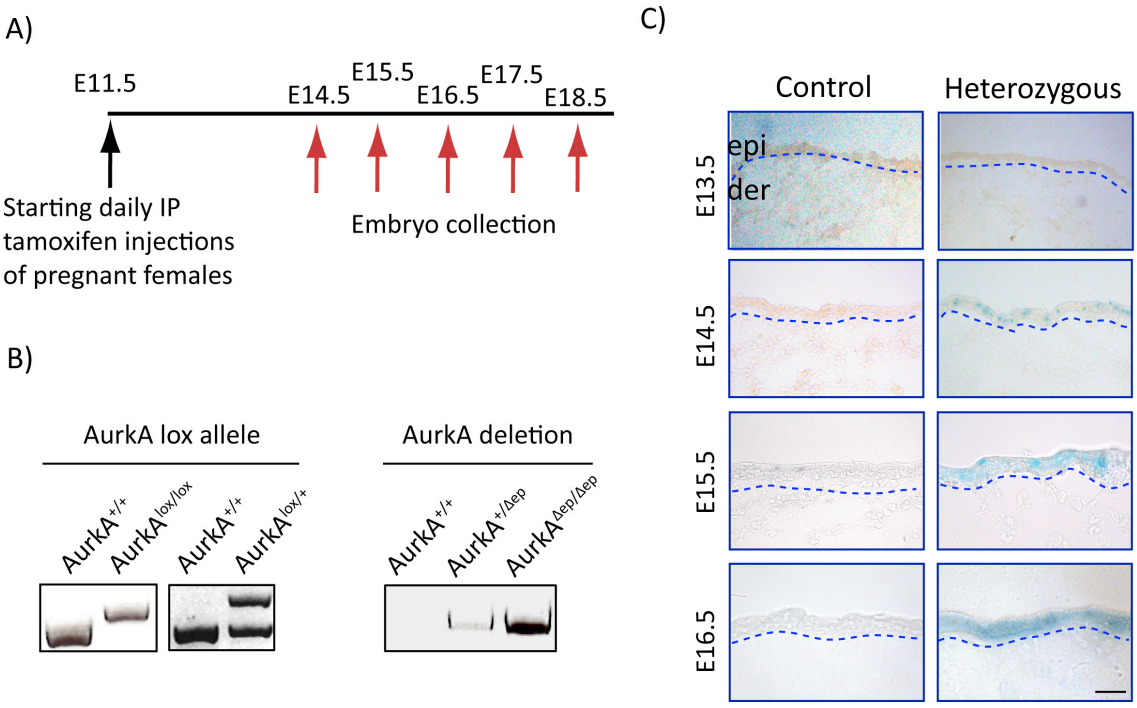


Figure 3 Analysis of the occurrence of genetic recombination events in the K14CreERT2$^{+/T}$; AurkA$^{\Delta ep/+}$ mice during skin development. A) Schematic representation of the protocol for the ablation of *AurkA* in mouse embryos. Daily tamoxifen injections were given to pregnant females, starting from E11.5 dpc, until the day of embryo collection. **B)** PCR analysis of the lox and deleted lox alleles in the embryos after the administration of tamoxifen. **C)** Presence of β-galactosidase activity in the AurkA$^{lox/+}$ control and K14 CreERT2$^{+/T}$; Rosa26LacZ$^{T/T}$ (heterozygous) embryos induced by tamoxifen. The blue color indicates the presence of β-galactosidase activity, which started appearing at E14.5. (n= 4 mice, 2 separate litters) epi: epidermis, der: dermis. Scale bar: 20 μm.

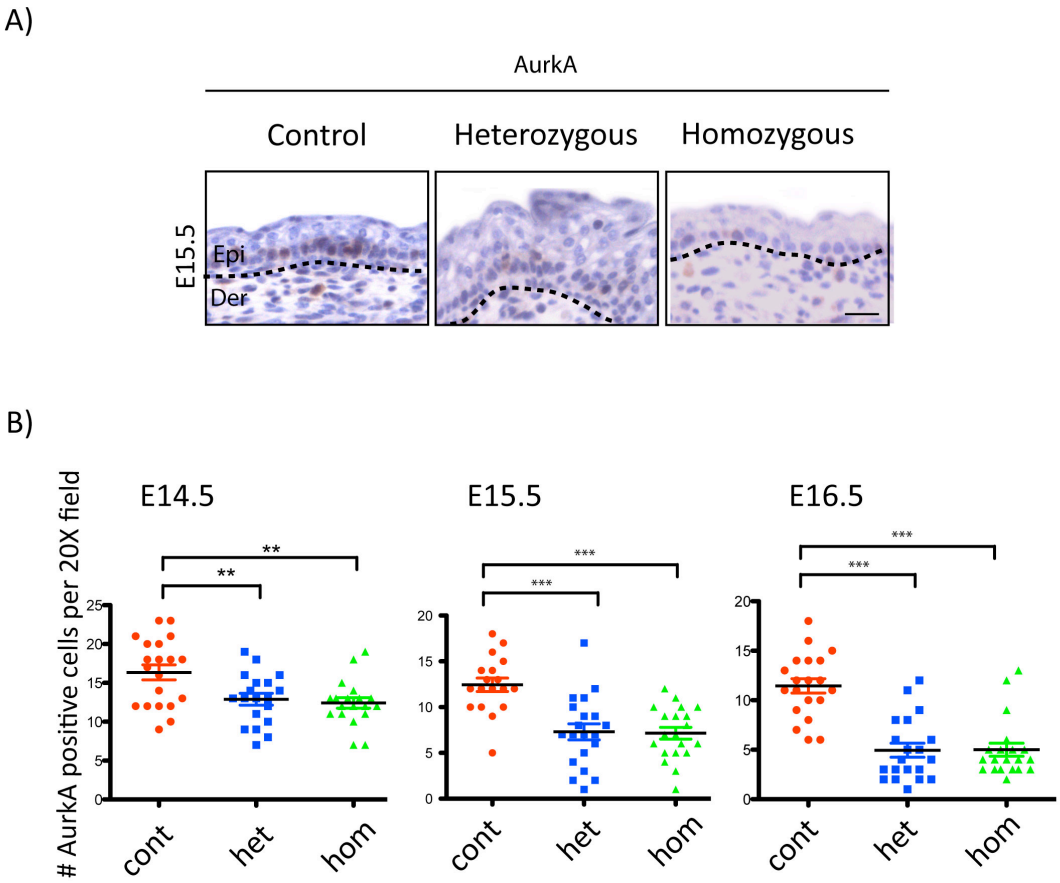


Figure 4 AurkA levels are reduced in the mouse embryo epidermis after tamoxifen administration. A) Representative images of the immunohistochemical analyses performed in in the epidermis of AurkA$^{lox/lox}$ (control), AurkA$^{\Delta ep/+}$ (heterozygous), and AurkA$^{\Delta ep/\Delta ep}$ (homozygous) mice at day E15.5, after 5 daily tamoxifen injections of pregnant females. epi: epidermis, der: dermis. Scale bar: 100µm **B)** Quantification of AurkA positive cells in the epidermis of control, heterozygous, and homozygous epidermises at E14.5, E15.5, and E16.5. For each experimental group, AurkA positive cells in the epidermis were counted. Data represents numbers of positive cells per 20x fields (n=3 embryos from different litters, 8 20x fields each). cont: control, het: heterozygous, hom: homozygousE14.5 **p= 0.0083, 0.0022. E15.5 and E16.5 ***p>0.0001.

1.3. Heterozygous loss of *AurkA* in the developing epidermis does not result in any apparent macroscopical defects, but leads to a transitory hyperplastic phenotype in the epidermis

Macroscopic analysis of E14.5, E15.5 and E16.5 embryos with heterozygous *AurkA* loss in the epidermis revealed the absence of any obvious macroscopic defects (Figure 5A). As expected, the *AurkA* null embryos in skin exhibited a translucent skin, with areas of skin loss, as well the absence of whiskers and eyelids. This was in agreement with the previous documented observations in embryos with *AurkA* null epidermis, using K14Cre transgenic mice, in which Cre is expressed in a constitutive manner (Torchia et al., 2013)

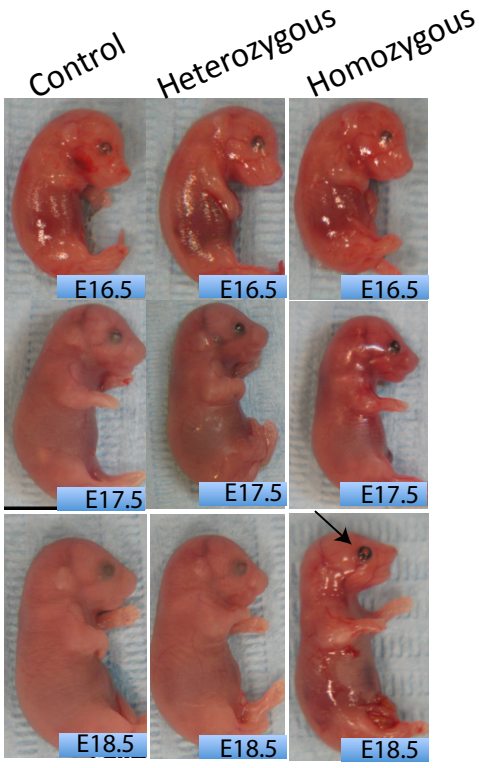


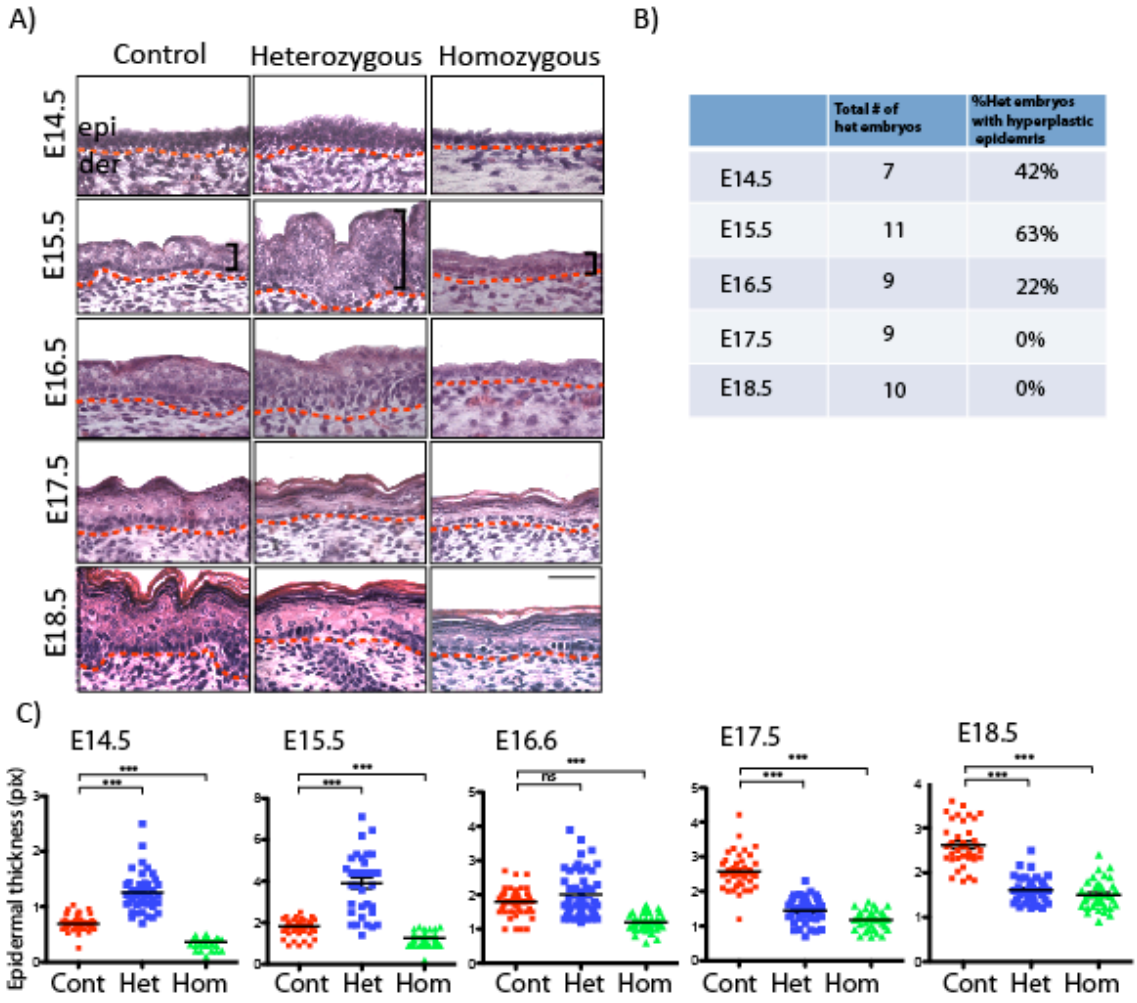
Figure 5 Heterozygous loss of *AurkA* in skin during epidermal development does not result in any apparent macroscopical defects. Macroscopic images of $AurkA^{lox/lox}$ (control), K14CreERT2; $AurkA^{\Delta ep/+}$ (heterozygous), and K14CreERT2; $AurkA^{\Delta ep/\Delta ep}$ (homozygous) embryos at different developmental stages. The arrow denotes the absence of eyelids in the conditional AurkA null embryos in skin. n= 3.

To examine more closely the consequences of the loss of AurkA in the developing epidermis, we performed Hematoxylin and Eosin (H&E) histological analyses of embryos with AurkA heterozygous and homozygous loss in skin (E14.5 to E18.5). Strikingly, the AurkA heterozygous epidermis at E14.5, E15.5, and E16.5 exhibited patches of

hyperplastic areas distributed across the epidermis, growing as columnar units (Figure 6A), which decreased as skin development progressed. As expected, the AurkA homozygous loss in the epidermis lead to a hypoplastic phenotype compared to controls (Figure 6A). Among all embryos with AurkA heterozygous loss in skin, 64% of E15.5 and 22% of E16.5 presented the hyperplastic skin phenotype, which was not observed at later developmental stages (Figure 6B). The quantitative analyses of the average of the global epidermal thickness of E15.5 and E16.5 AurkA heterozygous skin, revealed a trend in the increase across the whole epidermis, which was statistically significant (Figure 6C). Since progenitor cells are distributed in columnar units, these results suggests a defect in the columnar expansion of progenitor cells in the epidermis (Ghazizadeh and Taichman, 2001)

1.4 Heterozygous loss of *AurkA* in the developing epidermis leads to a transitory increase in the proliferation of basal progenitor cells

We hypothesized that the hyperplastic phenotype observed in AurkA heterozygous epidermis was due to a proliferation and differentiation misbalance of epidermal progenitor cells. To test this hypothesis, we first analyzed the levels of proliferation at the three developmental stages in which hyperplasia was observed (E14.5, E15.5, and E16.5). To this end, IP injections of BrdU were given to pregnant females 3 h before collecting the embryos. This was followed by IF analysis of BrdU in skin. The proliferation levels were determined by counting cells with incorporated BrdU (Figure 7A).



	Total # of het embryos	%Het embryos with hyperplastic epidermis
E14.5	7	42%
E15.5	11	63%
E16.5	9	22%
E17.5	9	0%
E18.5	10	0%

Figure 6 The heterozygous AurkA epidermis exhibits a transitory hyperplastic phenotype at early developmental stages A) H&E histological analyses of the AurkA$^{lox/lox}$ (control), K14CreERT2; AurkA$^{\Delta ep/+}$ (heterozygous), and K14CreERT2; AurkA$^{\Delta ep/\Delta ep}$ (homozygous) mice epidermis, at 5 consecutive days of development. Black brackets indicate a representative hyperplastic area in the E15.5 AurkA heterozygous epidermis. epi: epidermis, der: dermis, Scale bar: 40μm, n=between 7 and 10 embryos) **B)** Total number of embryos with heterozygous loss of AurkA in the skin collected, and the percentage of the number of embryos exhibiting the hyperplastic skin phenotype. **C)** Quantification of epidermal thicknesses across different areas across the backskin of embryos (n=3 embryos, 4 20x fields per embryo, 5 measurements per field):***p< 0.0001.

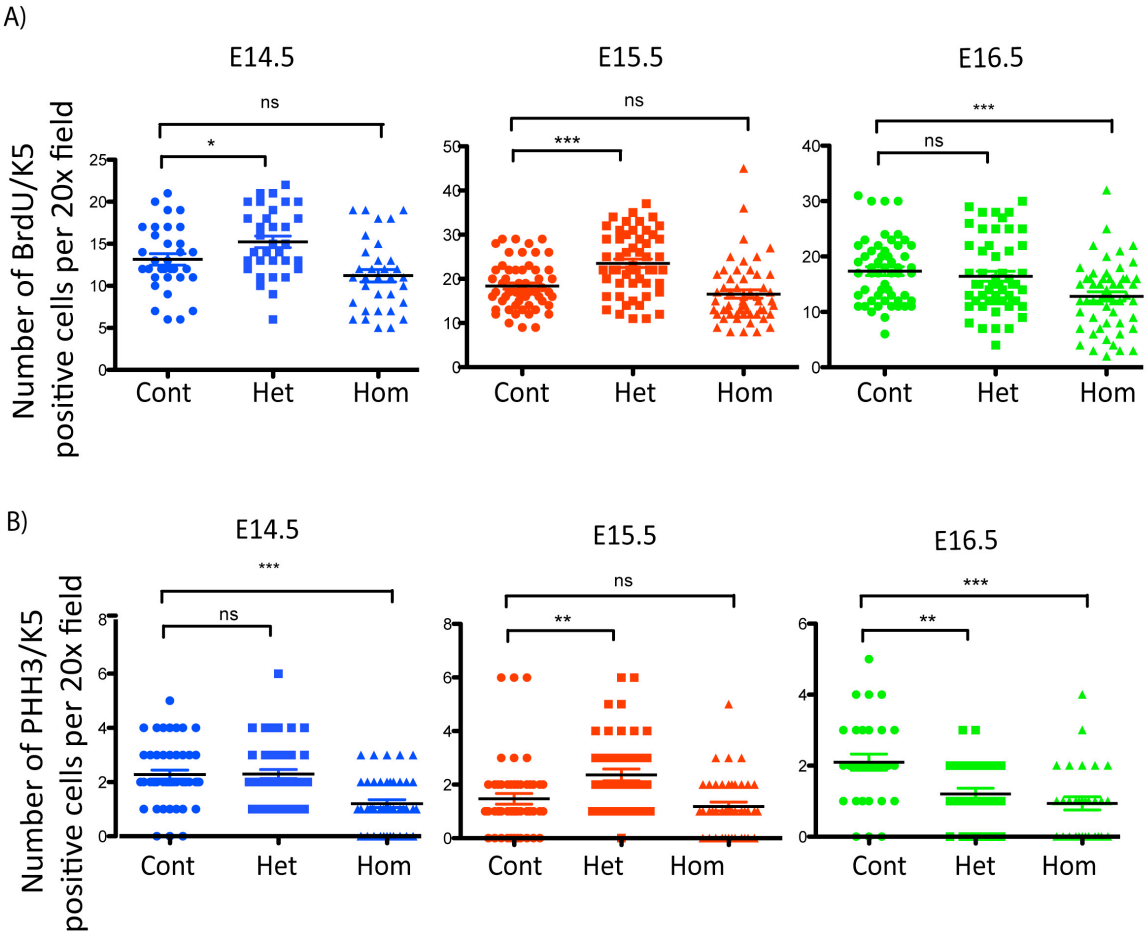


Figure 7 The heterozygous loss of AurkA in the epidermis leads to an increase in cell proliferation at early stages of epidermal development A) Quantification of BrdU/K5 positive cells in the epidermis of AurkA$^{lox/lox}$ (cont), K14CreERT2;AurkA$^{\Delta ep/lox}$ (het), and K14CreERT2;AurkA$^{\Delta ep/\Delta ep}$ (hom) embryos. Pregnant, tamoxifen treated females were injected with 100ug of BrdU 3 h before the embryo collection. Frozen embryo sections were then immunostained for BrdU and K5 and, so that only the basal, proliferating cells with incorporated BrdU can be counted. n=30 20X fields of the backskin of 3 different embryos, per experimental group, E14.5: *p=0.034; E15.5 and E16.5:***p< 0.0001 **B)** Quantification of PHH3/K5 positive cells, using the same embryos and quantification method as used for BrdU. E14.5:***p< 0.0001, E15.5:**p=0.0033; E16.5:**p=0.0023, ***p< 0.0001.

The quantification revealed a significant increase of the BrdU/K5 positive cells in the AurkA heterozygous epidermis at E14.5 and E15.5, while as expected, the homozygous loss of AurkA in epidermis led to a significant decrease, compared to controls.

To further validate these results, we performed Phospho Histone H3 (PHH3) stainings, to label cells in mitosis. Notably, consistent with our BrdU analyses, at E15.5 AurkA heterozygous epidermises also presented an elevated mitotic index, which decreased as skin development progressed (Figure 7B).

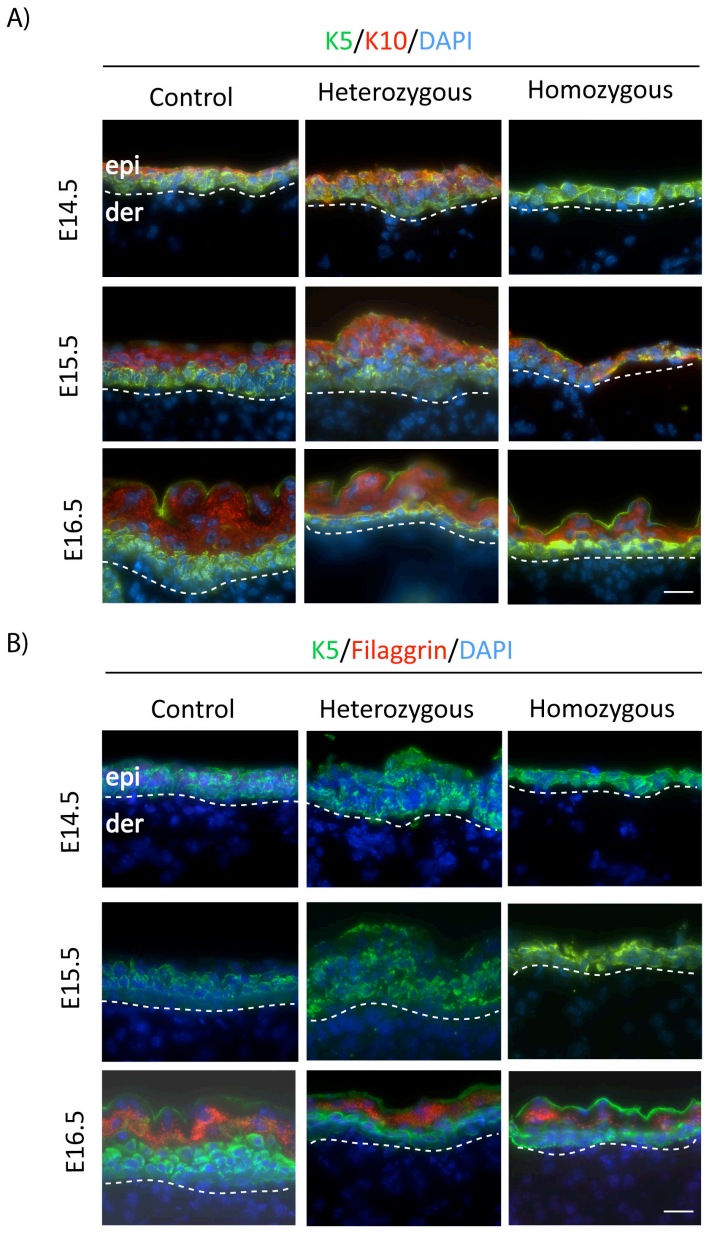


Figure 8 The heterozygous AurkA epidermis exhibits expanded areas of basal and differentiated layers **A)** Representative IF images of epidermises co-stained with the basal marker K5 and spinous marker K10 **B)** Representative images of epidermises costained with the basal marker K5 and late-differentiation marker Filaggrin in AurkA$^{lox/lox}$ (control), K14CreERT2;AurkA$^{\Delta ep/lox}$ (heterozygous), and K14CreERT2; AurkA$^{\Delta ep/\Delta ep}$ (homozygous) embryos at E14.5, E15.5, and E16.5. epi: epidermis, der: dermis. Scale bar: 20 μm.

To test whether the AurkA heterozygous epidermis also exhibited alterations in the terminal differentiation program, we performed IF analysis of differentiation markers.

The basal progenitor layer marker K5 as well as the spinous layer marker K10 were slightly expanded in areas of hyperplastic columnar growth (E14.5 and E15.5). Of note, there were no significant differences in the intensity of the fluorescent signal. Consistent with the previously documented phenotype, the AurkA null epidermis exhibited a hypoplastic phenotype with alterations in stratification and a delay in epidermal differentiation (Torchia et al., 2013), compared to controls (Figure 8A).

Interestingly, no alterations in the expression of the later differentiation marker Filaggrin at the corresponding E16.5 skin developmental stage were observed in the AurkA heterozygous epidermis, compared to their control counterparts. These results suggest that despite the increase in proliferation, the differentiation program was not delayed, probably to maintain the proliferation/differentiation balance to sustain skin homeostasis (Figure 8B).

Globally, these results suggest that the heterozygous loss of AurkA in the epidermis leads to an increase in cell proliferation shortly after its reduction at early developmental stages. This wave of proliferation seems to be later balanced by a proper differentiation program of skin.

2. Investigate the role of AurkA in the regulation of apical- basolateral polarity in epidermal cells, both *in vivo* and *in vitro*

2.1. Distribution of phospho Aurora kinase A (pAurkA) in mouse epidermis during development and in cultured primary keratinocytes

The transient hyperplastic phenotype observed in the AurkA heterozygous embryo epidermis was greatly intriguing. So far, no studies have shown that partial loss of AurkA in skin can lead to hyperproliferation, which seemed not to be due to the accumulation of genomic instability. Therefore, we decided to explore this novel observation.

While analyzing the differentiation status of the hyperplastic epidermis, we found that the hyperplastic areas were not simply an expansion of the epidermis, but the epidermis also appeared to be often disorganized, with different epidermal layers intermingled (Figure 9A). Similar phenotypes have been observed in *Drosophila* epithelia, when certain polarity proteins were depleted. Epithelial tissues of these flies not only presented hyperplasia, such as in the case of other tumor suppressors, but also a loss of the epithelial architecture (Bilder, 2004). Additionally, due to the previously published implications of AurkA in the process of cell polarity, we decided to further explore the possibility of AurkA playing a role in the regulation of the epithelial apical-basolateral polarity properties of epidermal cells, which could perhaps explain the observed phenotype *in vivo*.

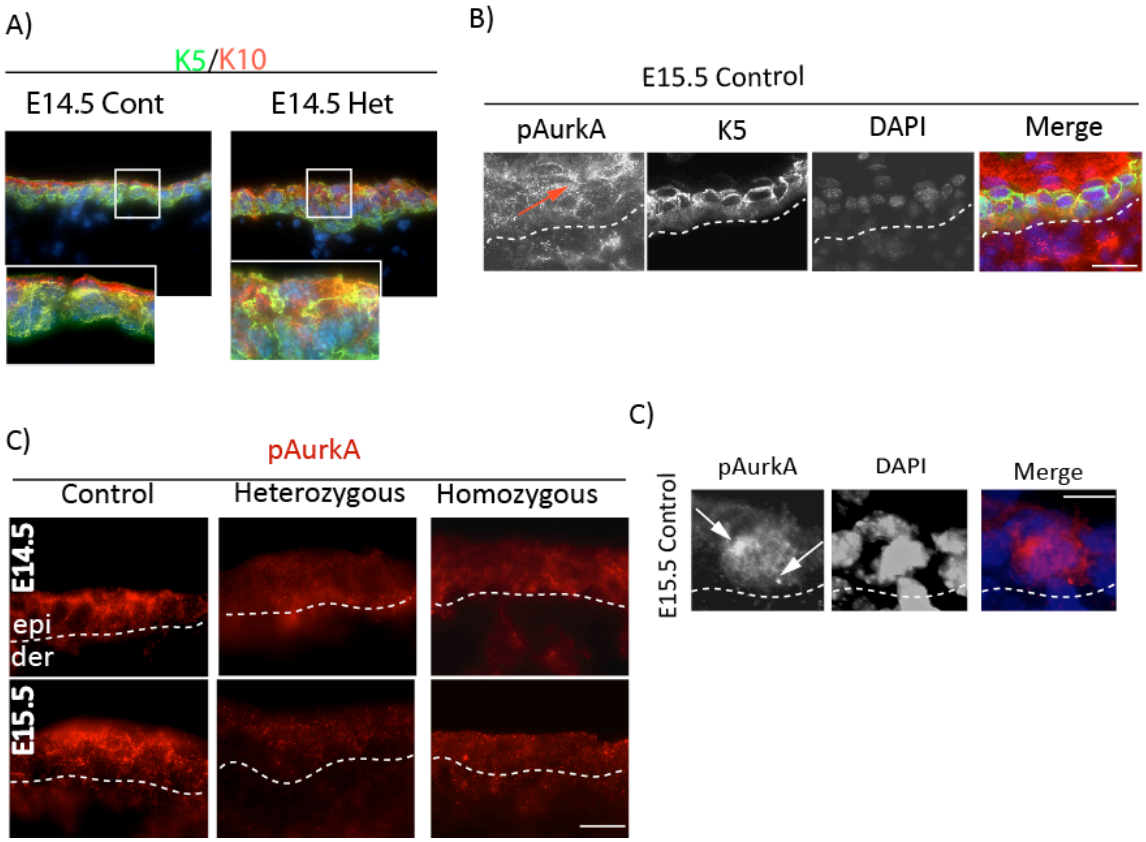


Figure 9 pAurkA is expressed at the cell cortex in both basal and differentiated layers of the embryo epidermis A) Representative images of the immunofluorescence co-staining for K10 and K5 with magnification insets showing the intercalation of the basal and suprabasal layers in AurkA heterozygous embryo epidermis **B)** Immunofluorescence co-stainings of pAurkA and the basal layer marker K5 in control E15.5 epidermis. The arrow indicates the predominant suprabasal distribution of pAurkA. Scale bar: 20 μm **C)** Immunofluorescence staining of pAurkA in the epidermis of $AurkA^{lox/lox}$(control), $K14CreERT2;AurkA^{\Delta ep/+}$ (heterozygous), and $K14CreERT2;AurkA^{\Delta ep/\Delta ep}$ (homozygous) embryos at E14.5 and E15.5. epi: epidermis, der: dermis. Scale bar: 20 μm **D)** Representative image of a mitotic cell in the basal layer, stained for pAurkA in E15.5 epidermis. The arrows point to the centrosome, a bona-fide localization of pAurkA during cell division. Scale bar: 10 μm.

To examine where active AurkA localizes in the skin, we performed IF stainings for the active form of AurkA, phospho AurkA (pAurkA), both in the epidermis and in keratinocytes growing *in vitro*. In control epidermis, the IF signal of pAurkA was present in the apical area of basal progenitor cells, and around the whole cell cortex in epidermal postmitotic differentiated layers, suggesting a non-mitotic role of AurkA (Figure 9B).

Intriguingly, this localization mirrors that of the polarity proteins aPKC and Par3 in skin. Also, there was a significant reduction of the signal in the case of all the mutants, indicating that the pAurkA signal is specific (Figure 9C).

Next, we investigated the localization of pAurkA in keratinocytes growing *in vitro*. As observed *in vivo*, pAurkA localized to the centrosomes in dividing keratinocytes. We later stimulated the differentiation of keratinocytes with Ca^{++}, which also induces the formation of cell-cell junctions. After 24 h under these conditions pAurkA localized to the cell cortex (Figure 10A and B). To further validate the novel finding of the cortical localization of AurkA, we transfected differentiated and non-differentiated keratinocytes with GFP-AurkA and GFP-control constructs, and we observed that the GFP-AurkA signal in differentiated keratinocytes was present at the cell cortex, while in undifferentiated keratinocytes it was found at the centrosomes of dividing cells (Figure 7C). These results, once again imply a non-mitotic role of AurkA in mouse keratinocytes.

To further validate the specificity of the AurkA signal in keratinocytes, we induced the ablation of AurkA with tamoxifen in growing K14CreERT2; $AurkA^{lox/lox}$ keratinocytes. The treatment led to a significant decrease of pAurkA at the cell cortex of differentiated keratinocytes (Figure 7D). We also treated the cells with the specific pAurkA inhibitor MLN8237-Alisertib for 12 h. This treatment caused a complete loss of the pAurkA signal at the cell cortex (Figure 7E). This treatment also led to a decrease in the number of mitotic cells, and an increase in DNA content, confirming the efficacy of the inhibitor (data not shown). All together, these results suggest that in mouse skin epidermis, as well as in *in vitro* keratinocytes, pAurkA is expressed at the cortex of non-proliferating cells, suggesting a possible non-mitotic role for AurkA.

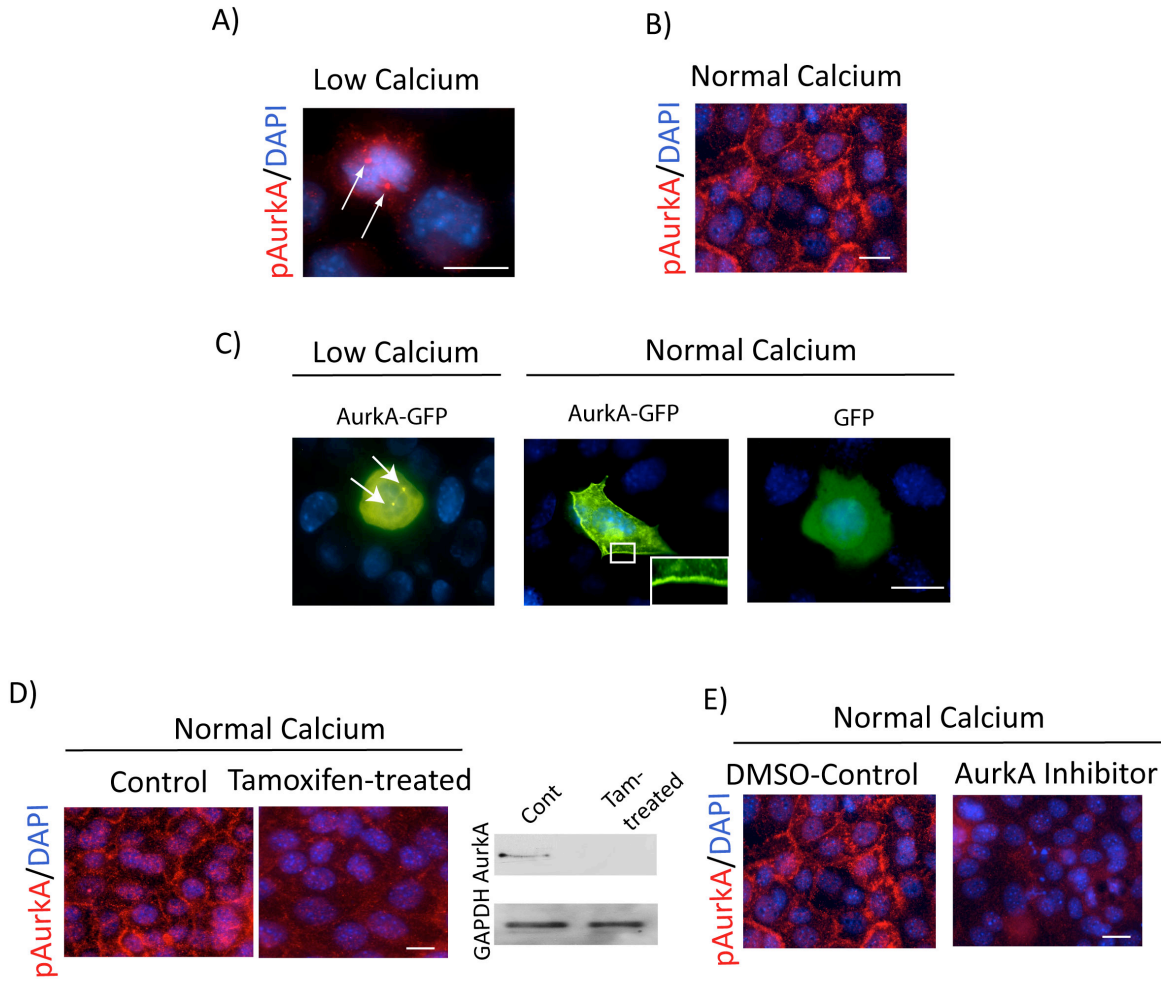


Figure 10 pAurkA localizes at the cell cortex in differentiated keratinocytes *in vitro*

A) IF analysis of the distribution of pAurkA in wild-type proliferating keratinocytes (arrows indicate the localization of the pAurkA signal at the centrosomes of a dividing cell. Scale bar: 10μm **B)** pAurkA in wild-type differentiated keratinocytes, where cells were grown in normal-calcium media for 12 h to induce differentiation **C)** A proliferating wild-type keratinocyte expressing AurkA-GFP at the centrosomes (indicated by the arrows. Wild-type differentiated keratinocytes expressing AurkA-GFP and GFP alone. The magnified insets indicate the cortical localization of AurkA-GFP **D)** pAurkA staining in control ($AurkA^{lox/lox}$) and tamoxifen treated (AurkA-/-) keratinocytes and the western blot showing the total AurkA levels after 24 h of tamoxifen treatment **E**) pAurkA distribution in differentiated wild-type and AurkA-inhibitor treated, keratinocytes. Scale bar: 20 μm.

2.2. AurkA deficiency or inhibition leads to the mislocalization of the apical-basolateral cell polarity proteins aPKC and Par3 both *in vivo* and *in vitro*

To test whether AurkA plays a role in the regulation of apical-basolateral polarity in the epidermis of developing embryos, we first examined the bona-fide markers of polarity, components of the Par complex, Par3 and aPKC (Figure 11 A and B). Consistent with previous results, the two proteins are localized apically in basal progenitor cells, and around the whole cell cortex of differentiated suprabasal cells. Interestingly, in AurkA heterozygous and null epidermis this localization was aberrant, and the signal was more diffused in the cytoplasm (Figure 11A and B), suggesting that AurkA may play a role in the proper distribution of these polarity determinants in the epidermis.

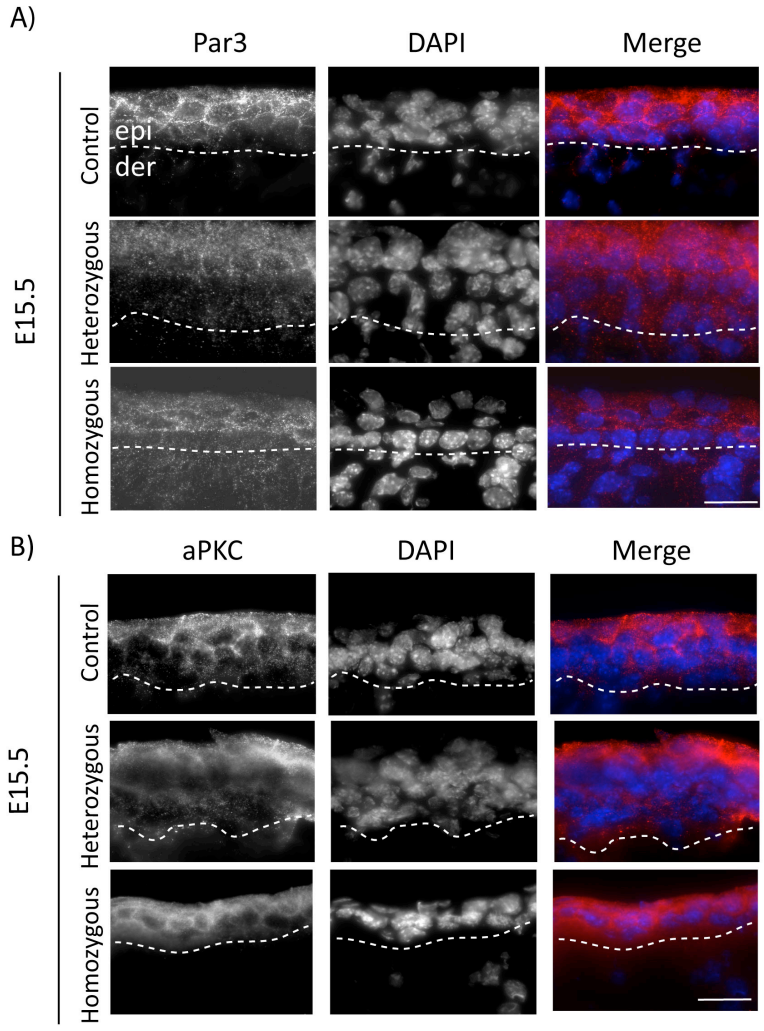


Figure 11 AurkA deficiency leads to the aberrant localization of the polarity proteins Par3 and aPKC in the embryo epidermis

A) Representative images of epidermal IF stainings of the apical proteins Par3 and **B)** aPKC in the AurkA$^{lox/lox}$ (control), K14CreERT2;AurkA$^{\Delta ep/lox}$ (heterozygous), and K14CreERT2;AurkA$^{\Delta ep/\Delta ep}$ (homozygous) embryos at E15.5. epi: epidermis, der: dermis Scale bar: 20 μm.

To test whether these events were cell autonomous, we analyzed the localization of

aPKC and Par3 in K14CreERT2; AurkA$^{lox/lox}$ primary keratinocytes upon treatment with tamoxifen or with the MLN8237-Alisertib pAurkA inhibitor (Figure 12A and B). The loss of inhibition of AurkA led to the disruption of the cortical localization of these two polarity proteins (Figure 12A and B). This effect was not only due to alterations in the distribution of these proteins, but also to a decrease in their protein levels as observed by immunoblot analysis (Figure 9 C).

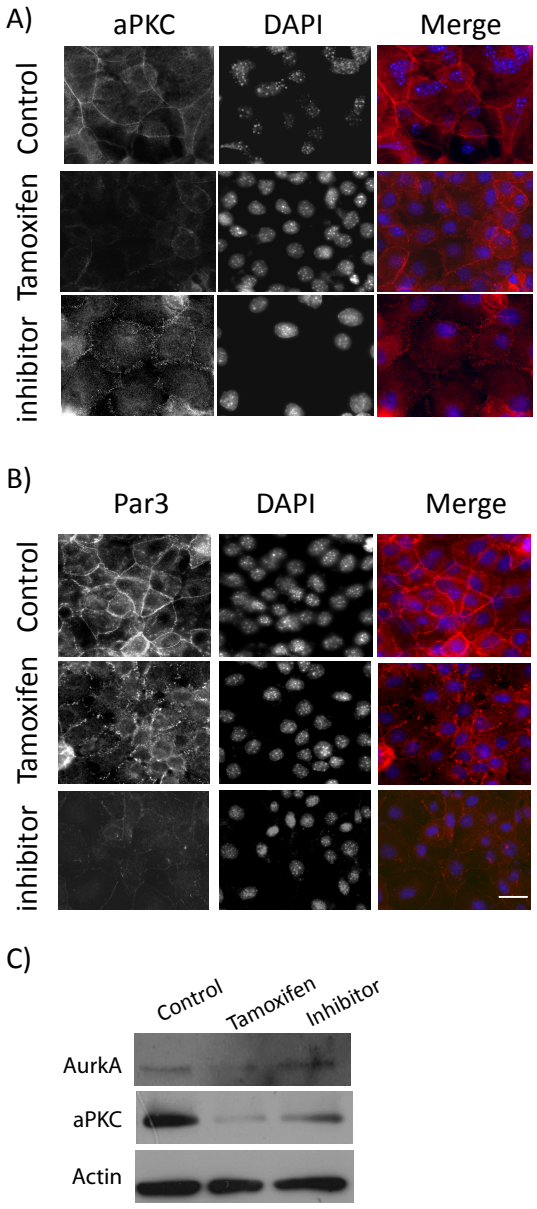


Figure 12 Loss or inhibition of AurkA in vitro leads to the mislocalization and reduction in the protein levels of the polarity proteins Par3 and aPKC in differentiated keratinocytes

A) Representative images of the IF analysis of aPKC and **B)** Par3 in differentiated keratinocytes treated with either tamoxifen or the pAurkA inhibitor. Cells were treated with tamoxifen for 24 h or with the AurkA inhibitor for 12 h, under Low Calcium conditions, followed by the addition of NC media supplemented with either tamoxifen or the AurkA to trigger cell differentiation. 24 h later cells were fixed and analyzed. Scale bar: 40 μm . **C)** Biochemical analysis of Par3 and aPKC proteins, using the lysates of the cells treated using the protocol described above.

2.3. AurkA deficiency or inhibition perturbs AJ and TJ formation

Some of the key components that work in concert with the cell polarity machinery are AJs and TJs. As mentioned in the introduction, studies have shown that polarity proteins and the junctions are important for each other's functions. Therefore, as we hypothesize that AurkA may behave as a polarity protein in the epidermis; we decided to study its involvement in the formation of AJs and TJs. We first evaluated the distribution of AJs proteins in AurkA heterozygous and AurkA null embryo epidermis. For this, we stained for ECAD, a transmembrane protein of the AJ complex (Figure 13). In both AurkA heterozygous and homozygous epidermis, ECAD was more diffused, compared to the cortical localization observed in controls. Moreover, the signal was significantly reduced in the basal layer of the epidermis, indicating possible aberrations in AJ formation and/or stability. Interestingly, the phenotype observed was not only restricted to the hyperplastic patches of the heterozygous embryos, but was also present in areas that were not hyperplasic.

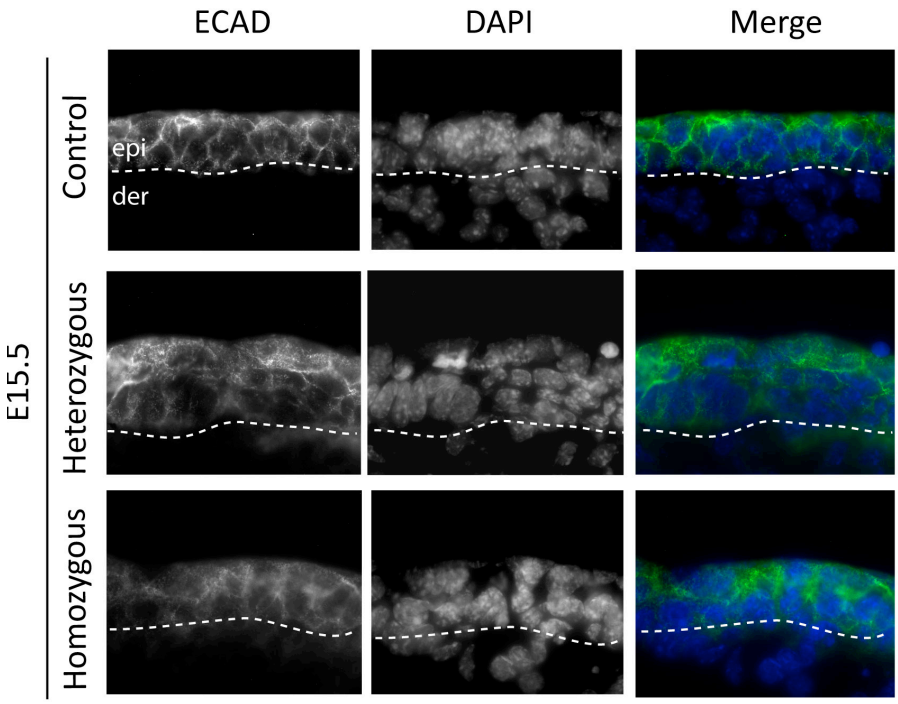


Figure 13 Heterozygous and homozygous AurkA embryo epidermis presents aberrant localization of ECAD Representative images of IF analysis of ECAD in the epidermis of AurkA$^{lox/lox}$ (control), K14CreERT2;AurkA$^{\Delta ep/lox}$ (heterozygous), and K14CreERT2;AurkA$^{\Delta ep/\Delta ep}$ (homozygous) embryos at E15.5.

To investigate if the loss of AurkA caused alterations in the establishment of AJs, we turned to *in vitro* studies. To this end, we performed calcium switch experiments, where the addition of normal calcium media induces AJ formation in confluent keratinocytes (Figure 14A and B). In control cells, after 24 h of calcium switch, AJs were already formed, as denoted by the presence of ECAD at the cell-cell contacts. However, tamoxifen or AurkA inhibitor treated cells exhibited a significant delay in the formation of AJs (Figure 14A and B). Biochemical analyses showed that after 12 h of calcium switch both tamoxifen and inhibitor treated cells had significantly decreased levels of ECAD, as compared to controls (Figure 14C).

Next, we analyzed the consequences of the loss or inhibition of AurkA in TJ formation, using the tight junction marker Occludin. We observed that both AurkA deficient and AurkA inhibited cells were unable to form TJs (Figure 15A). Interestingly, however, unlike ECAD, the protein levels of Occludin were not changed compared to controls (Figure 15B). It is well established that defects in TJs lead to the loss of the epithelial barrier. For this reason, we wanted to test whether the AurkA heterozygous and null embryo epidermis presented skin barrier defects. To this end, we performed permeability barrier assays, and as expected, both embryos with heterozygous and homozygous loss of AurkA in the epidermis exhibited a severe barrier defect (Figure 16).

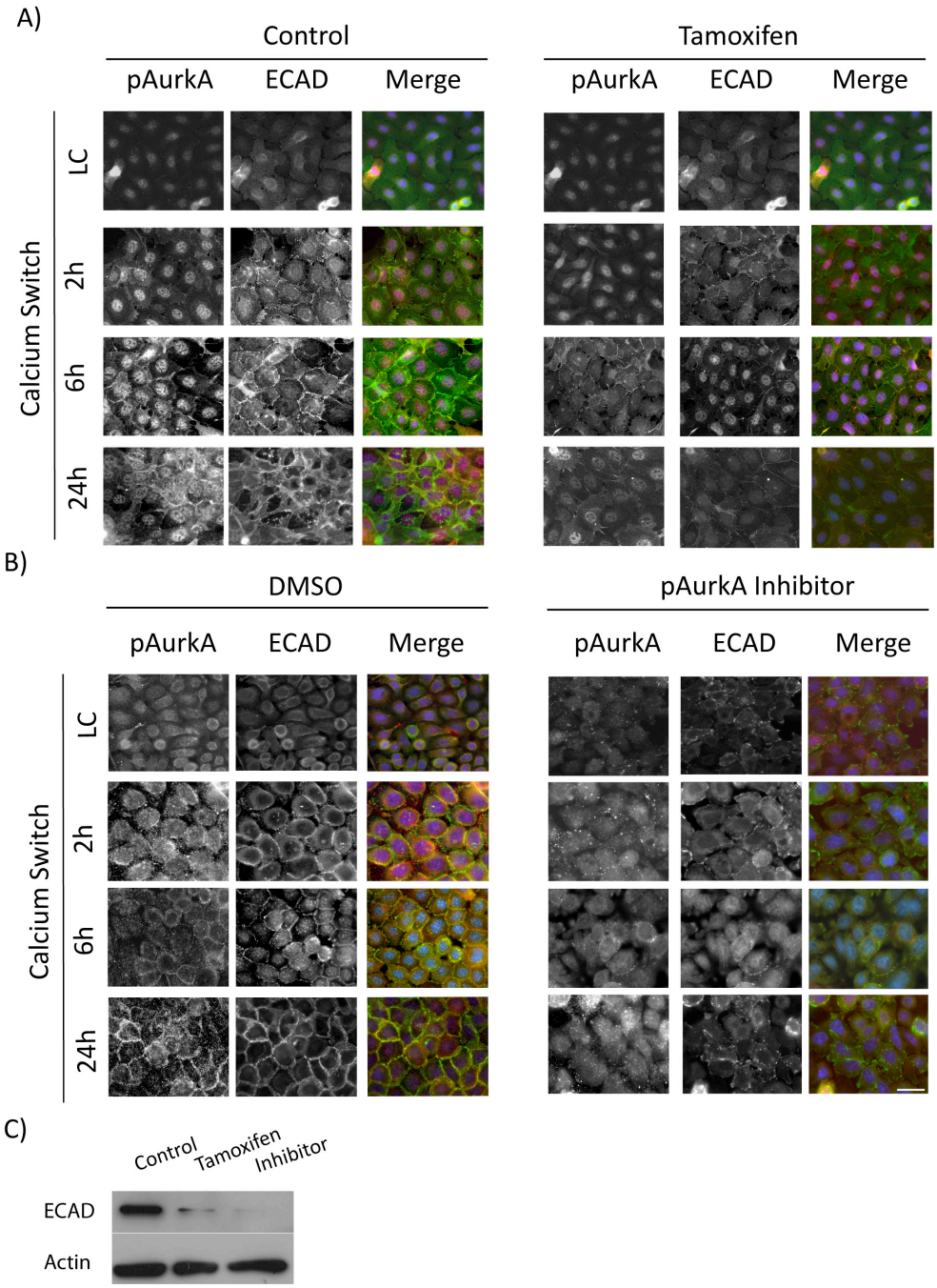


Figure 14 Loss or inhibition of AurkA leads to a delay in AJ formation and downregulation of ECAD A) Representative images of IF analysis of ECAD of cells pretreated with the AurkA inhibitor or DMSO as control for 12 h, followed by calcium switch in the presence of the treatments and analyzed after 0.5 h, 2 h and 12 h **B)** IF analyses of ECAD in cells pretreated with tamoxifen for 12 h and analyzed at different time points after calcium-switch in the presence of tamoxifen (1 h, 6 h, and 24 h) **C)** Biochemical analysis of ECAD using the lysates of the cells treated using protocols described above, after 24 h of calcium switch. Scale bar: 40 μm.

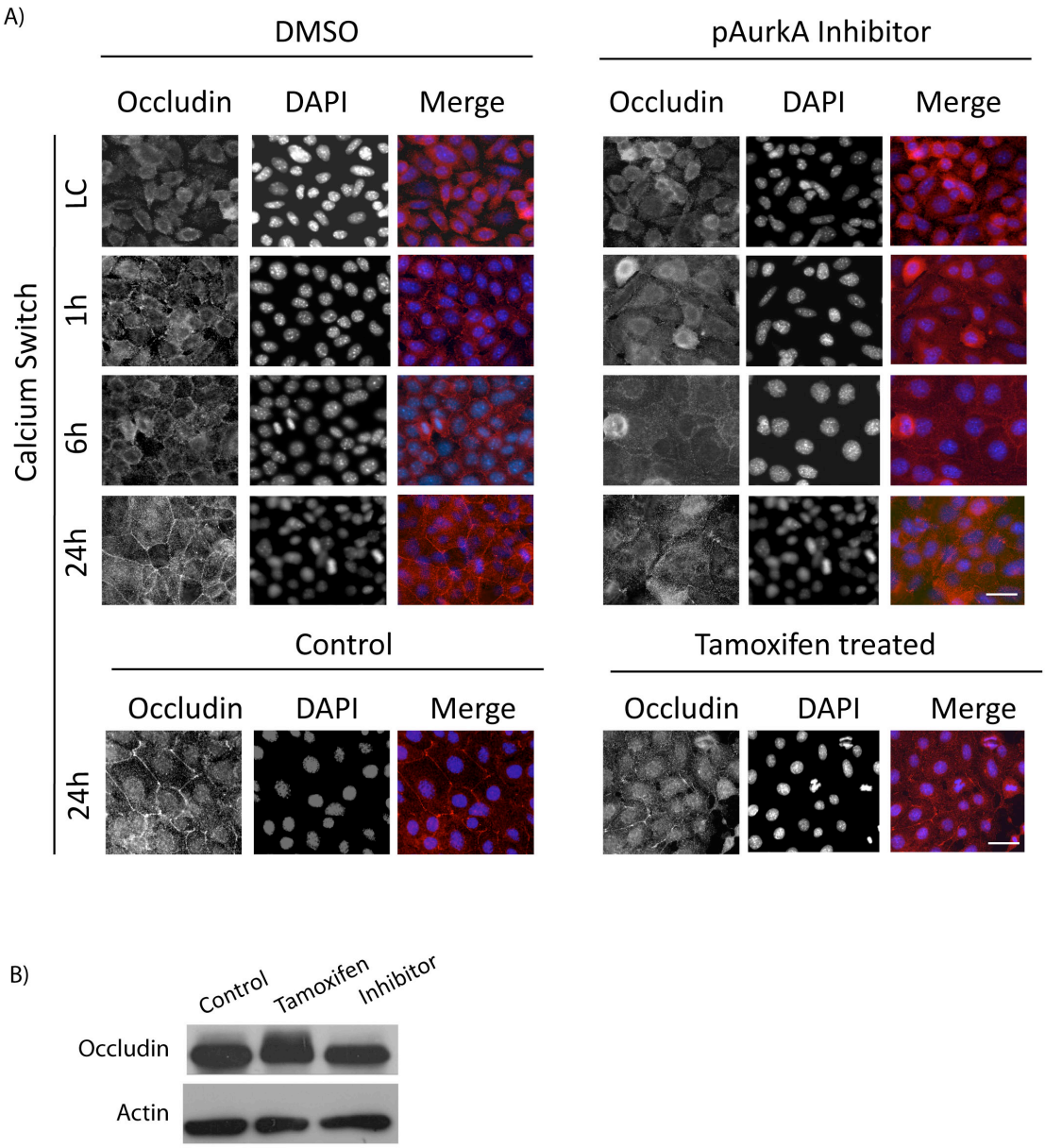


Figure 15 AurkA loss or inhibition leads to tight junction formation delay *in vitro*

A) Representative images of the IF analysis of Occludin after the calcium switch experiment, where control (DMSO) and inhibitor or tamoxifen treated cells were added normal calcium media for different lengths of time (1 h, 6 h, and 24 h), after being treated with tamoxifen for 24 h or inhibitor for 12 h. **B)** Biochemical analysis of E-cad using the lysates of the cells treated using the same protocol as described above. Scale bar: 40 μm

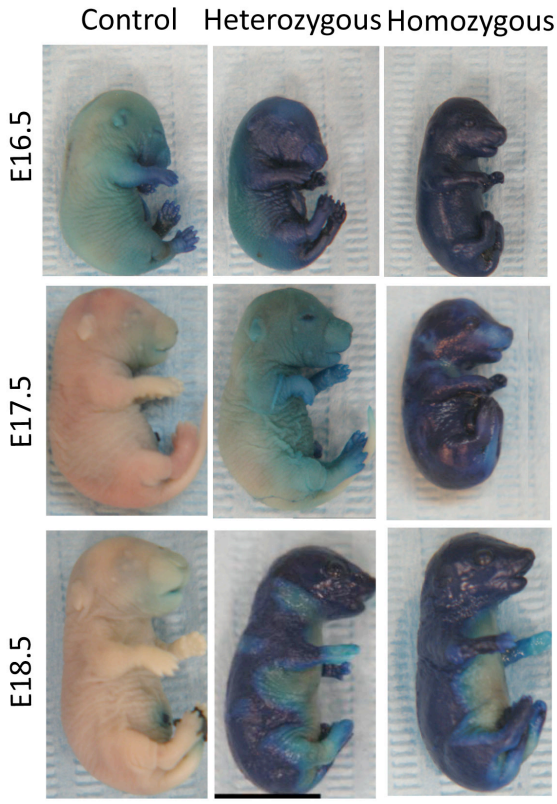


Figure 16 AurkA deficiency in the epidermis leads to barrier defects In the barrier-permeability assay, the blue color indicates the penetrance of the bromophenol blue dye through the skin. Note the dye penetrance in embryos with AurkA deficient skin, at E17.5 and E18.5, in which the barrier should have already been formed (n=4 embryos).

2.4. AurkA deficiency leads to defects in orientation of the spindles of dividing cells

The establishment and maintenance of apical-basolateral polarity is tightly related to the orientation of cell divisions. To explore this, we monitored the presence of dividing basal progenitor cells and the orientation of their cell divisions by costaining embryo epidermises with K5 and PHH3 (Figure 17A and B). Parallel and perpendicular cell divisions were counted in both controls and AurkA heterozygous epidermis in E14.5 and E15.5 embryos that presented the hyperplastic phenotype. Only the AurkA heterozygous epidermises were quantified, since the AurkA null epidermis exhibited extremely low number of PHH3 positive cells. For the quantifications, only the cells with clear parallel or perpendicular orientation, in relation to the basement membrane were considered. Quantification of parallel vs. perpendicular divisions revealed that in the E14.5 AurkA heterozygous epidermis there was an increase in the occurrence of perpendicular cell divisions, which could explain the early expansion of differentiated layers (Figure 8A). In

E15.5 embryos, we observed an increase of parallel cell divisions, which could explain the bias towards symmetric cell divisions and the expansion of the proliferative layer. It is important to note that the ratios between parallel and perpendicular divisions in the control E14.5 and E15.5 embryos are consistent with previously published observations (Lechler and Fuchs, 2005).

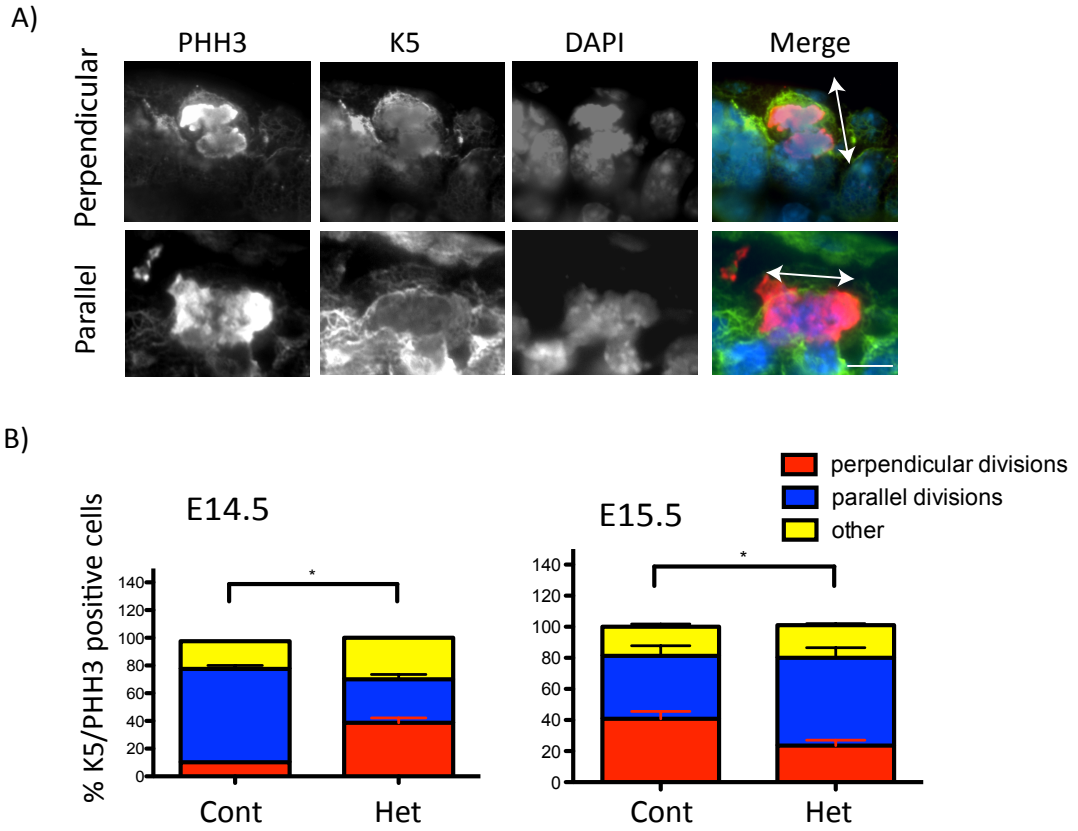


Figure 17 E14.5 and E15.5 AurkA heterozygous embryo epidermises present misoriented cell divisions A) Representative images of cells dividing parallel and perpendicular to the basement membrane, as indicated by the arrows. PHH3 staining is used to visualize the orientation of the plane of cell division and K5 staining was used as a reference for the division angle. Scale bar: 5μm **B)** Quantification of the parallel, perpendicular, and all other orientation divisions in the epidermis of AurkA$^{lox/lox}$ (control) and K14CreERT2;AurkA$^{\Delta ep/lox}$ (het) embryos (E14.5 and E15). PHH3/K5 positive cells in the backskin epidermis that exhibited a clear orientation of the plane of cell division were counted. All PHH3/K5 positive cells were counted and categorized into those dividing parallel to the basal membrane, perpendicular to the basal membrane, and all the other orientations. Significance values refer to the comparison of the perpendicular division fraction p= 0.0140, (n=3, approx. 40 cells counted).

2.5. Mechanisms by which AurkA may regulate apical-basolateral polarity

So far we have demonstrated that loss of AurkA leads to loss of cell polarity *in vivo* and *in vitro*. Polarity markers, such as Par3 and aPKC, as well as AJs and TJs were disrupted when AurkA levels are reduced or its functional activity is inhibited. To decipher the hierarchy of these defects, we first explored what happens when aPKC is inhibited. To this end, we treated differentiated keratinocytes with a specific aPKC inhibitor. Under these conditions, pAurkA remained localized at the cell cortex, indicating that it functions upstream of the Par complex, as demonstrated in other systems (Figure 18A). However, there was a delay in the formation of AJs, similar to the one observed in the absence of AurkA, although not as severe (Figure 18B), suggesting that the Par complex is upstream of AJs formation in keratinocytes. Next, to explore whether AJs can also modulate the cortical localization of pAurkA, we analyzed its localization in ECAD KO; PCAD shRNA keratinocytes (Tinkle et al., 2008). First, we confirmed by IF analysis that the cells were negative for both ECAD and PCAD (Fig. 19A and B), and that were also unable to form TJs, as published before (Tinkle et al., 2008) (Figure 19A). Interestingly, we observed that in the absence of ECAD and PCAD, pAurkA was unable to localize to the cell cortex after calcium switch. This suggests that pAurkA localization is dependent on cell-cell adhesion events. Together, these data suggest that pAurkA and cell junction formation are codependent, and that the Par complex proteins are part of this interaction.

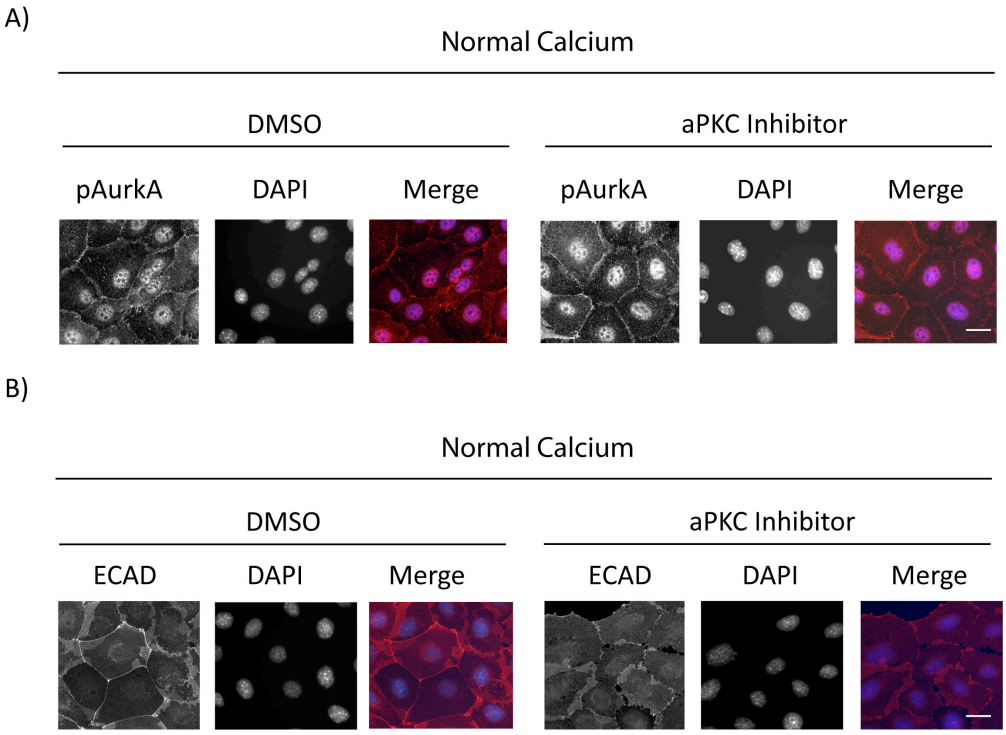


Figure 18 The inhibition of aPKC does not affect the cortical localization of pAurkA, but leads to a delay in AJ formation A) Representative images of IF stainings of pAurkA and **B**) ECAD localization in keratinocytes after treatment with 1μM aPKC inhibitor for 9 h, before differentiating them in normal calcium media for 24 h in the presence of the aPKC inhibitor, Scale bar: 40 μm.

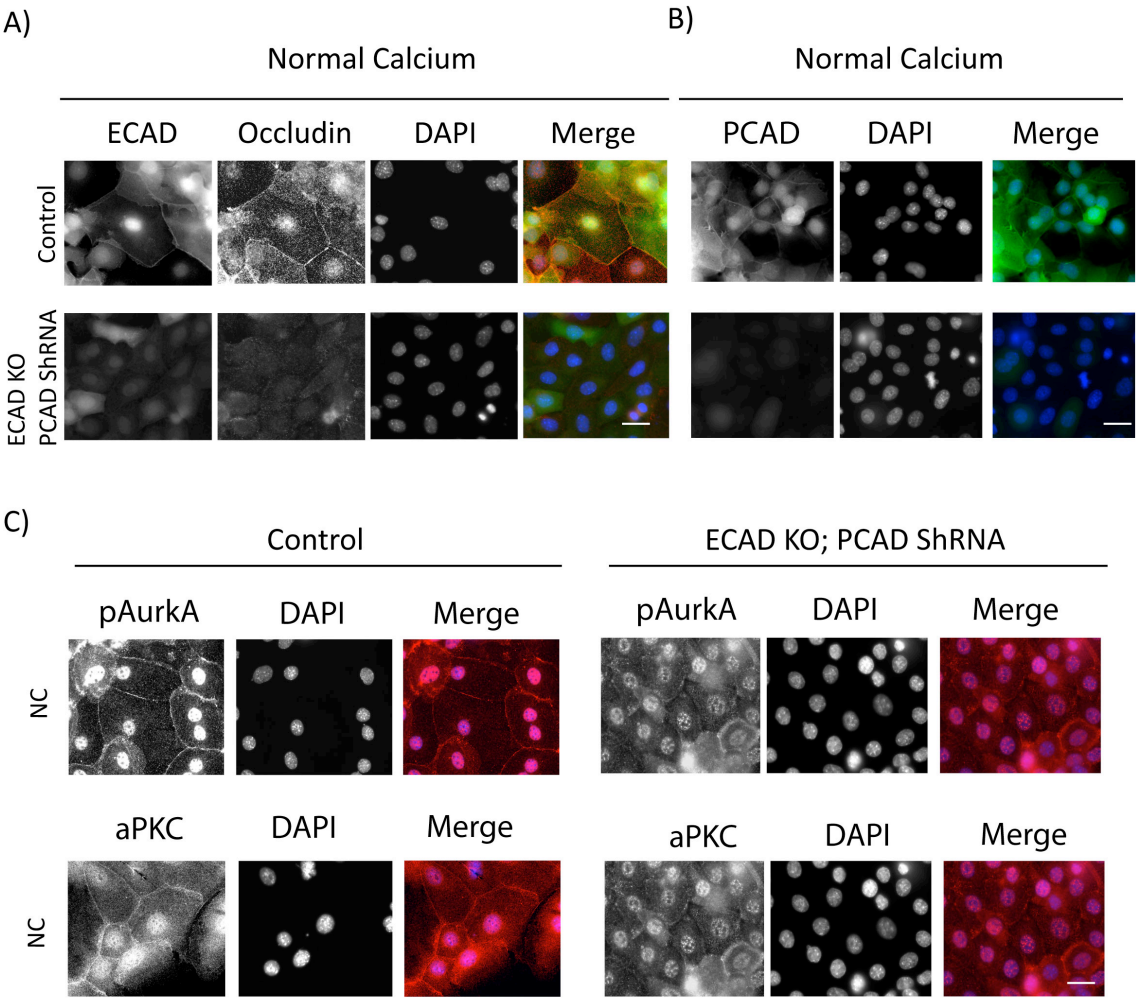


Figure 19 The cortical localization of pAurkA is dependent on AJ formation A) Representative images of IF stainings of ECAD and Occludin in control and ECAD KO; PCAD shRNA keratinocytes, Scale bar: 40 μm **B)** PCAD IF analysis in control and ECAD KO; PCAD shRNA keratinocytes **C)** Control and E-cad KO; P-Cad shRNA cells stained for pAurkA and aPKC after differentiation. For all stainings cells were differentiated in NC media for 24 h before fixation, Scale bar: 40 μm.

2.6. AurkA also regulates additional aspects of cell polarity in epithelial keratinocytes

Many polarity protein complexes have evolved to play a role during different kinds of cell polarity. In order to test whether AurkA may also behave in a similar way, we explored the effects of the AurkA deficiency on the front-rear polarity, a type of polarity important for the directional movement of cells. To address this question, we performed a scratch assay, where a scratch was make onto a monolayer of confluent keratinocytes, and the keratinocytes were allowed migrate to fill in the 'wound'. 9 h later, migrating keratinocytes should have acquired front-rear polarity. As a result of this polarity, organelles, such as Golgi become oriented towards the leading edge, facing the 'wound'. Therefore, after the scratch assay we stained the Golgi and counted the number of cells at the leading edge with oriented (towards the leading edge) and misoriented (any other direction) organization (Figure 20A). AurkA heterozygous keratinocytes presented an increase in the number of cells with misoriented Golgi, suggesting a problem in cell polarization during migration (Figure 20B). To confirm the observed phenotype, we performed a cytoochip experiment where cells are plated on fibronectin micropatterns to which they attach and acquire an 'umbrella' shape that forces the cells to acquire a front-rear polarity, that mimics the situation during cell migration (Figure 10C). Similar to the scratch assay, we stained the Golgi and counted the number of control and AurkA heterozygous keratinocytes that exhibited an oriented or misoriented Golgi (Figure 20D). Interestingly, there was also a significant increase in the number of AurkA heterozygous keratinocytes displaying a misoriented-Golgi, compared to controls. The two experiments could not be performed with the AurkA null keratinocytes, since replating the cells after treatment with tamoxifen led to a great cell loss.

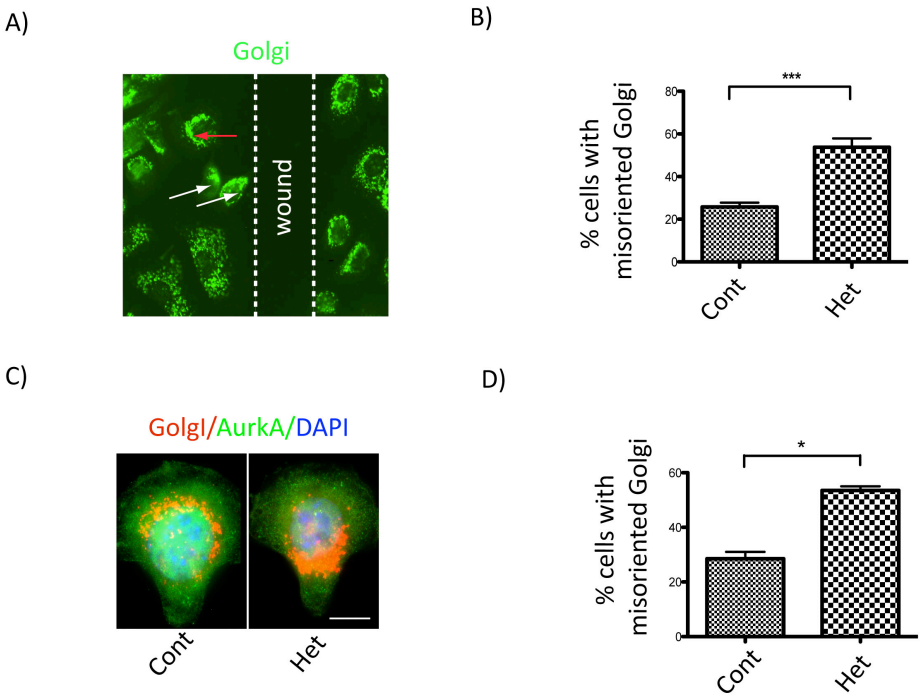


Figure 20 AurkA heterozygous keratinocytes exhibit aberrations in front-rear cell polarity *in vitro* A) Representative images of IF analysis of the Golgi, 9 h after a scratch was made onto the cell monolayer. White arrows denote a properly oriented Golgi towards the leading edge, and the red arrow a cell with a misoriented Golgi **B)** Quantification of keratinocytes at the leading edge with misoriented Golgi. AurkA$^{lox/+}$ (control) and AurkA$^{-/+}$ (heterozygous) cells (n=3 experiments). ***,p< 0.0001 **C)** Representative images of keratinocytes plated on cytoochips coated with fibronectin, stained with a Golgi marker and for AurkA. Scale bar: 5μm **D)** Quantification of cells presenting a misoriented Golgi in control and heterozygous cells.*p=0.0188.

2.7. Deficiency of AurkA also leads to apical-basolateral polarity defects in the gold standard MDCK model of cell polarity

Our studies have uncovered a previously unappreciated role of AurkA in the regulation of the apical-basolateral polarity of epidermal cells. To validate that this role is also observed in other cellular systems, we took advantage of the gold-standard cell model system of apical-basal polarity, MDCK cells. First, we analyzed if pAurkA also distributed to the

cell cortex in MDCK cells (Figure 21A). We then performed knock down (KD) approaches to reduce the levels of AurkA by transfecting MDCK cells with a cocktail of two siRNAs specific for AurkA, in addition to include transfections with scramble controls. Upon AurkA KD, the signal of pAurkA at the cell cortex of MDCK cells was significantly reduced. The same was observed when the cells were treated with the specific AurkA inhibitor (Figure 21B).

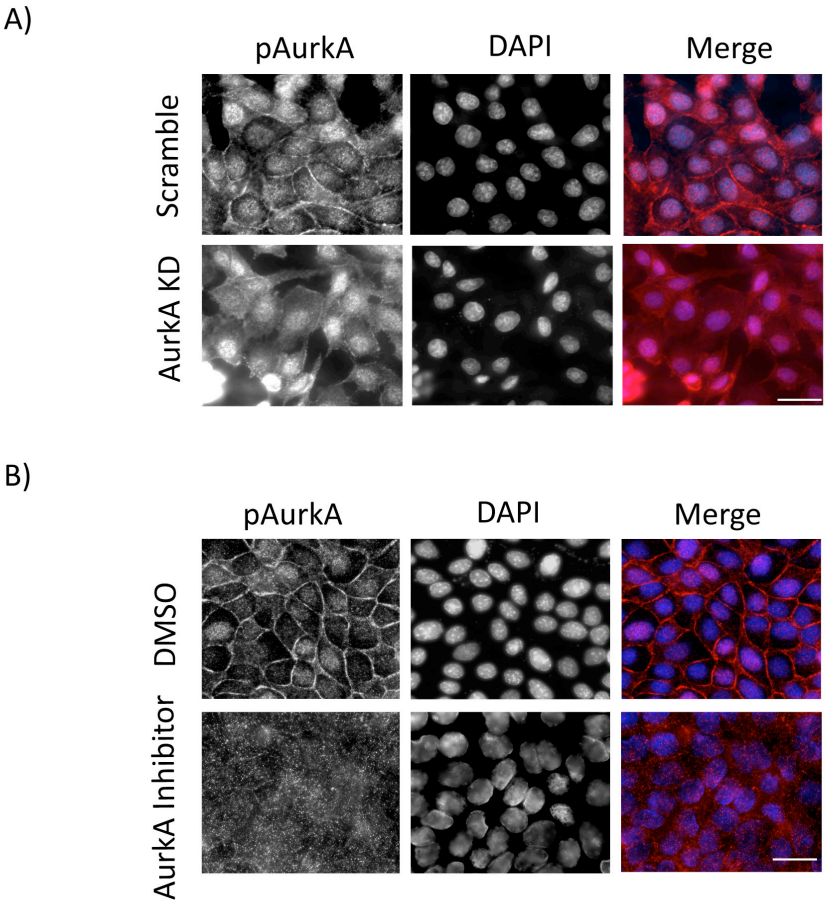


Figure 21 pAurkA localizes at the cell cortex of MDCK cells A) Representative images of IF stainings for pAurkA in control and AurkA KD MDCK cells. **B**) pAurkA staining in control (DMSO) and MDCK cells treated with the pAurkA inhibitor for 12 h. Scale bar: 40 μm.

To test if AurkA deficiency leads to aberrations in the localization of proteins of the Par complex, we analyzed the distribution of aPKC and Par3 in both AurkA-KD and AurkA inhibitor-treated cells (Figure 22A and B). Reductions of AurkA led to the mislocalization of aPKC from the cell cortex. However, interestingly it did not seem to affect the localization of Par3.

We also took advantage of the 3-D MDCK cysts model system, in which cells growing on matrigel are induced to form tubular structures with well-defined features of cell polarization. Since AurkA KD cysts were arresting in mitosis, and since we wanted to uncouple the mitotic defect from the polarity one, we treated late-stage cysts (day 4) with the specific AurkA inhibitor, for only 10 h (Figure 23A). In comparison to rounded control cysts displaying lumens, the inhibitor treated ones presented very irregular shapes and protrusions, in addition to an increase in the number of lumens. We were also interested to see if the loss of AurkA in MDCK cells also induced a delayed in AJ formation. To this end, we performed calcium switch experiments and observed a clear delay in the formation of AJs in AurkA KD cells when compared to controls (Figure 23A).

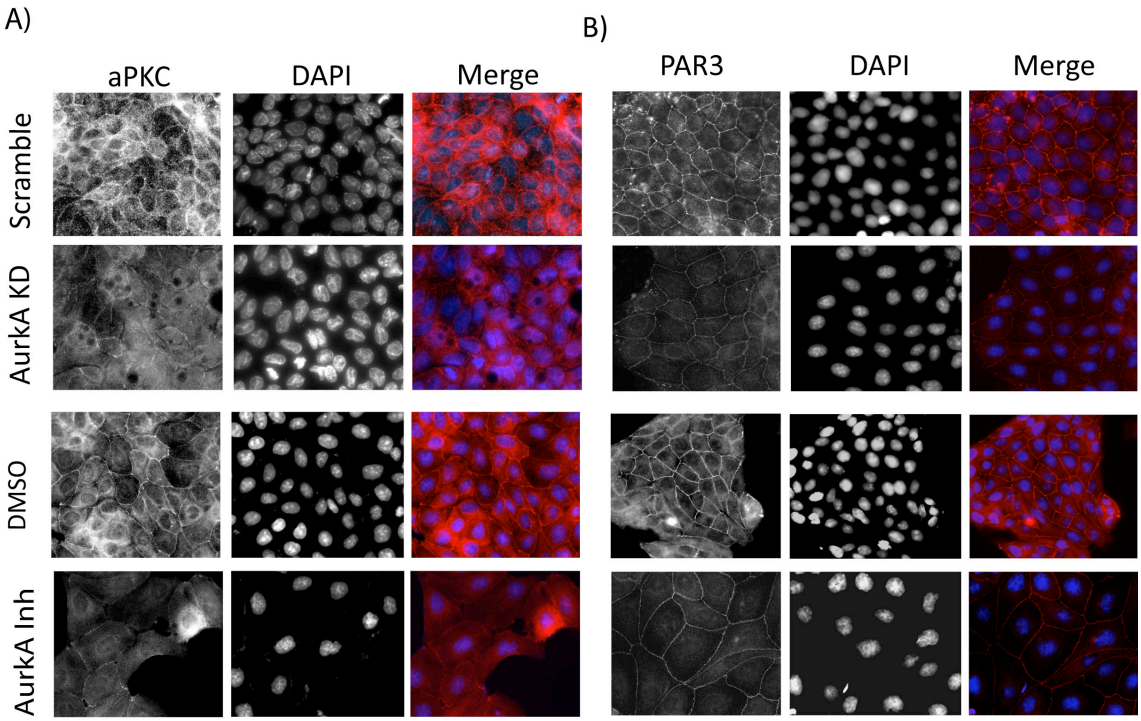


Figure 22 AurkA KD or inhibition of AurkA lead to the mislocalization of aPKC and PAR3 in MDCK cells A) Representative images of IF analysis of aPKC and **B**) Par3 in KD and inhibitor treated MDCK cells. Scale bar: 40μm

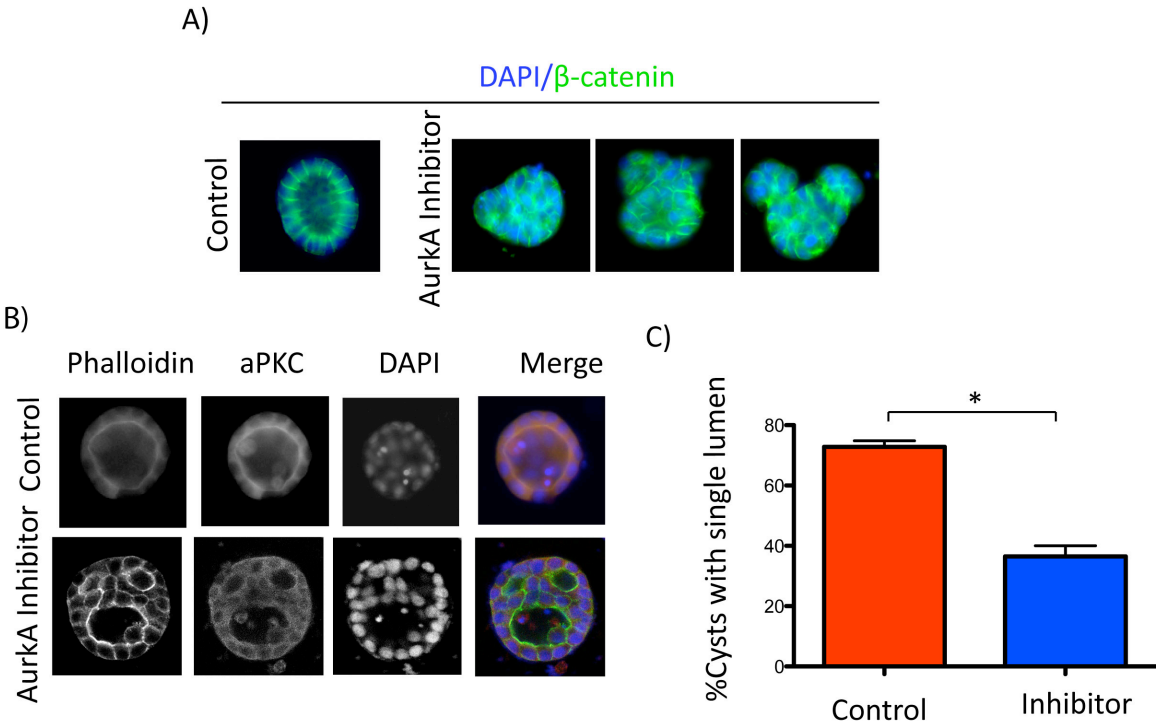


Figure 23 3-D MDCK cysts present an aberrant organization in the presence of an specific AurkA inhibitor A) Representative images of the control and inhibitor-treated MDCK cysts stained for β-catenin, which allows for the visualization of the cyst shape. Cysts were allowed to form for 72 h after plating the cells, followed by the addition of the AurkA for 18 h before fixation. **B**) IF staining for aPKC in control and inhibitor treated cysts. **C**) Quantification of the number of cysts with single lumens. *p=0.0121. Over 100 cysts were counted, n=2, number of experiments.

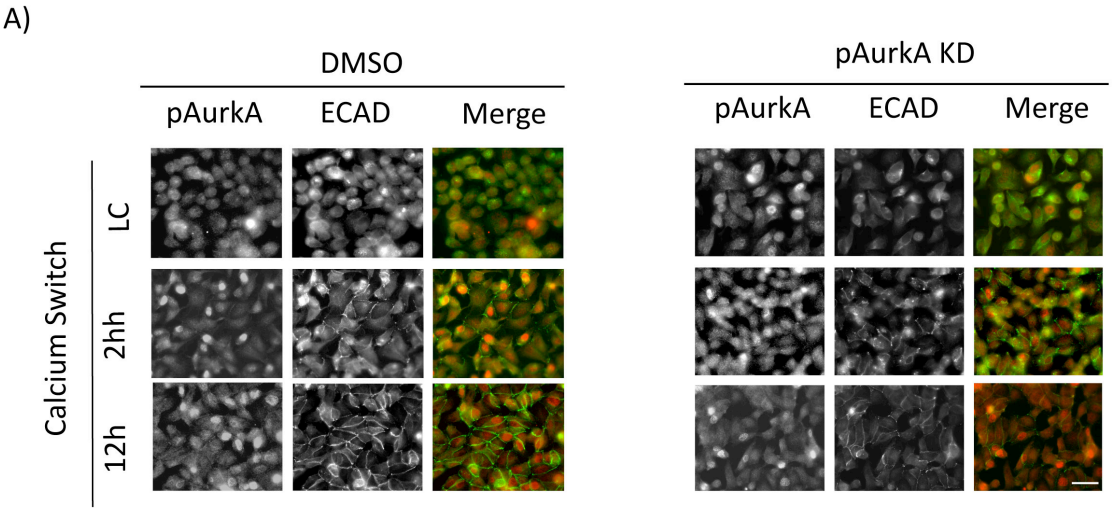


Figure 24 KD of AurkA in MDCK cells causes a delay in AJ formation. A) Representative images of IF analysis of ECAD after 2 h and 12 h of calcium switch, in control AurkA KD cells. Scale Bar: 40 μm.

2.8. Insights on the consequences of the heterozygous loss of AurkA in epidermal tumorigenesis

One of the interesting facets of our results is the requirement of fine-tuned levels of AurkA to sustain skin homeostasis. We observed that the heterozygous loss of AurkA in embryo epidermis led to a transient wave of hyperproliferation associated to alterations in cell polarity. This scenario differed from the one observed in embryos with homozygous loss of AurkA in the epidermis, which led to hypoplasia and a decrease in cell proliferation. The hyperplastic phenotype points to the possibility that partial losses of AurkA could possibly play a tumor-promoting role in the epidermis. We wanted to further explore this question. To this end, we performed the well-defined DMBA/TPA carcinogenesis protocol in the epidermis of adult control and AurkA heterozygous mice (Faraldo et al., 1997). Our preliminary results indicated a lower latency of appearance of papillomas in AurkA heterozygous epidermis when compared to controls, although fewer numbers of papillomas were observed (Figure 25). These preliminary results may resemble the more

physiological scenario of epidermal tumors, and point to the potential risk of only achieving partial reductions of AurkA in current anticancer therapies.

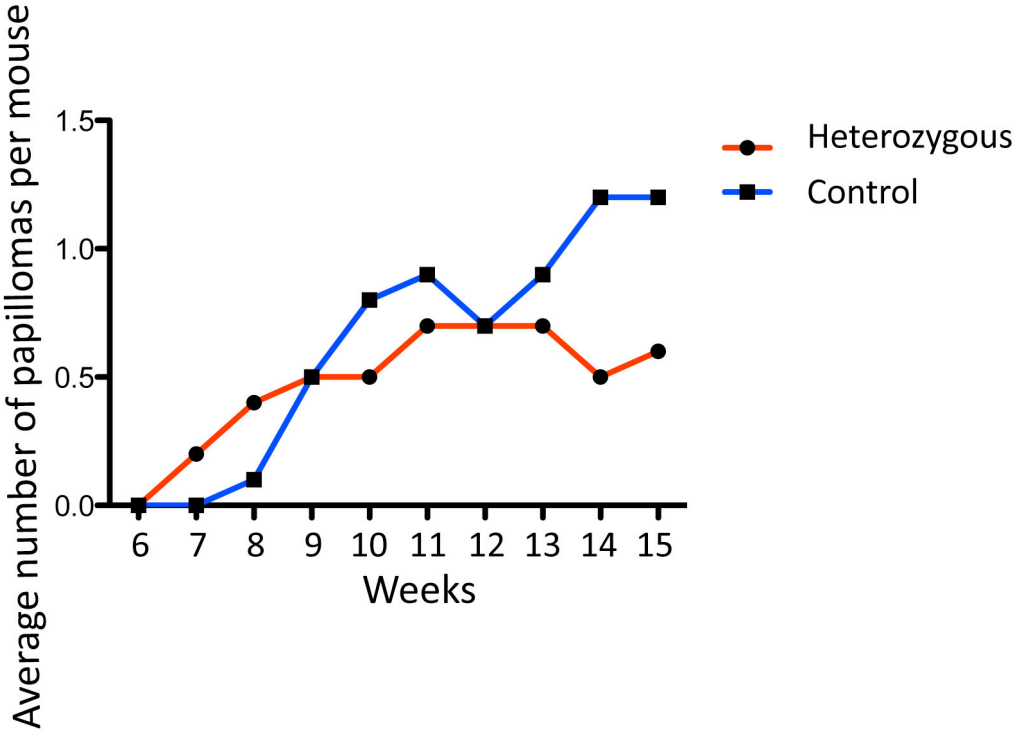


Figure 25 Heterozygous loss of AurkA in the epidermis leads to a lower latency of papilloma appearance upon administration of the DMBA/TPA carcinogenesis protocol Mice were injected with tamoxifen for 5 consecutive days, 3 days later DMBA was applied, followed by the administration of TPA twice a week during the duration of the experiment. n=2 experiments: 4 Heterozygous, 5 control mice.

Discussion

The question of how the architecture of a cell, tissue, or an organism dictates biological functions, and how biological functions can maintain tissue organization in a reciprocal manner, has been a long-lasting question for many scientists (Kirschner et al., 2000). Understanding this ying -yang relationship between biological structure and function will be fundamental to delineate how it underlies tissue homeostasis, and how when perturbed leads to disease stages or tissue aging. After years of research, it is becoming increasingly clear that changes in cellular shape and architecture can modulate tissue specific functions (Miller and Davidson, 2013; Guillot and Lecuit, 2013). However, the structure of the whole tissue or a cell depends on a well-engineered collaboration of different elements. In this grand design, cell polarity plays a very important role. Cell polarity refers to structurally, and thus functionally different domains within the cell. One type of polarity that has been greatly explored in eukaryotic models is apical-basolateral polarity in simple epithelia. Little is known, however, about this kind of cell polarity in more complex epithelial tissues, such as the stratified epithelial tissue of the skin, the epidermis. In this project we aimed to extend this knowledge, and with our findings we hope to have contributed to the understanding of the mechanisms that underlie apical-basolateral polarity in the epidermis. Our results uncovered a novel function of Aurora kinase A in the regulation of apical-basolateral polarity, which goes beyond its roles as a mitotic protein, and as a regulator of oriented cell divisions. The physiological role of AurkA in the context of apical-basolateral polarity has not been so far observed in other tissues. Our findings now open new possibilities for further examination of the precise roles of AurkA in the regulation of cell polarity and its implications for the maintenance of the physiological balance of epithelial tissues.

1. Beyond cell division: AurkA as a novel player in the establishment and maintenance of apical-basolateral polarity

AurkA has proved to be a protein with a variety of roles. It has been shown to play a role in cellular processes other than mitosis, including cell polarity. The emerging functions of

AurkA in cell polarity appear to be evolutionary conserved. In the lower eukaryote *Drosophila melanogaster*, it has been shown that it regulates asymmetric neuroblast divisions by interacting with the Par complex (Wirtz-Peitz et al., 2008). Moreover, the distribution of cell fate determinants between *Drosophila* neural progenitors and daughter cells also seems to be regulated by AurkA activity (Cayouette and Raff, 2002). Interestingly, virtually all cell polarity proteins, including those of the Par complex, are as well evolutionary conserved (Siller and Doe, 2009). In mammals, AurkA has only been recently associated with the regulation of spindle orientations of multipotent progenitor cells residing in adult tissues, such as the mammary epithelia (Regan et al., 2013).

In addition to this emerging role in oriented cell divisions, AurkA has also been implicated in the polarized growth of neurites, which facilitate the directional flow of signaling cues in neural cells (Yamada et al., 2010). Of note, this level of cell polarization, parallels apical-basolateral polarity of epithelial cells (Famulski and Solecki, 2013).
Despite the tantalizing roles that have emerged for AurkA from these studies, the functional importance of this protein in the regulation of epithelial apical-basolateral polarity remains unclear. Hence, we hypothesized that this protein also played a role in apical-basolateral polarity in the skin epidermis. To address this hypothesis we used a genetic mouse model of conditional deletion of AurkA in epidermal cells, both *in vivo* and *in vitro,* as well as the pharmacological inhibition of AurkA in *in vitro* keratinocytes.

AurkA and the Par complex

One of the most novel findings of this study is the distribution of pAurkA to the cell cortex of epithelial cells. Both *in vivo* and *in vitro*, pAurkA localized not only to the apical site of epidermal progenitor cells, but also to the cell cortex of postmitotic differentiated keratinocytes, further supporting the existence of additional roles for AurkA beyond its mitotic roles. Moreover, pAurkA localization mirrored the localization of the Par complex proteins, Par3 and aPKC, and deficiency or inhibition of AurkA led to the mislocalization of the two Par complex proteins. Strikingly, in out system AurkA regulated the stability of the two proteins. We believe these results would open a new avenue for investigating the mechanisms through which AurkA may regulate the Par complex in mammalian cells. It is tempting to speculate, that similar to the models

proposed in *Drosophila* (Wirtz-Peitz et al., 2008), and in neurons (Khazaei and Püschel, 2009), that AurkA can also function in apical-basolateral polarity via phosphorylation of Par proteins, allowing their distribution to specific polarized domains.

AurkA and junctional stability: physiological implications in skin barrier function

AJs and TJs are seminal components of the apical-basolateral polarity. On one hand, they contribute to sort the polarity complexes to their proper domains. On the other hand, some polarity proteins can be important for the maintenance of these adhesion complexes. Our results showed that deficiency or inhibition of AurkA led to delays in AJ formation, accompanied by a significant reduction of the protein levels of ECAD. Interestingly, keratinocytes mutant for both ECAD/PCAD fail to recruit pAurkA to the cell cortex. Together, these results indicate that upon differentiation, AurkA is necessary for the proper formation of AJs, and reciprocally, AJs are necessary for the cortical localization of AurkA. But how could AurkA regulate AJs? An interesting link that has been made in the previous studies is between Ajuba, an activator of AurkA, and ECAD (Nola et al., 2011). This study showed that loss of Ajuba led to Rac activation, which in turn destabilized cadherin receptors at the membrane. However, since loss of Ajuba did not lead to changes in the levels of ECAD, likely indicates the existence of additional mechanisms by which AurkA regulates ECAD protein stability.

Since AJs are necessary for the formation of TJs, and ECAD protein levels were reduced, it was not surprising to observe a delay in TJs formation and in the recruitment of Occludin to the cell cortex. Interestingly, the total levels of Occludin were not changed. Thus, it is possible that AurkA directly regulates the stability of ECAD, which in turn affects TJs. To further answer this question, it would be interesting to see through which mechanism AurkA stabilizes ECAD protein. A possible hypothesis would be that it directly or indirectly phosphorylates ECAD, which leads to its destabilization and degradation.

Next, we hypothesized that defect observed in TJs *in vitro* also occurs in the epidermis, in which the embryos with heterozygous and homozygous loss of AurkA in skin would have problems developing skin barrier, a process that normally starts at E16.5 (Hardman et al.,

1998). Indeed, we found that both heterozygous and homozygous embryos presented a severe skin barrier defect. However, curiously enough, the AurkA heterozygous skin at E17.5 are able to form a partial functional barrier, compared to controls, to later lose it by E18.5. Probably by the accumulation of defects including reductions in the functional activity of AJs and TJs. It would be interesting to further address AurkA's role, *in vivo* and *in vitro*, in the maintenance of apical-basolateral polarity and junction stability, after it has already been established.

Due to the severe defects observed in AurkA deficient embryos in skin, they were always sacrificed by the mothers upon birth, suggesting that their phenotype must likely be perinatally lethal. However, this finding is intriguing, since it has been shown that mice with constitutive, homozygous deletion of AurkA are embryonically lethal, whereas mice with heterozygous constitutive deletion of AurkA survive and live until late adulthood (Lin-Yo, 2008). It is possible that the germline deletion of one allele of AurkA allows the organism to develop a compensatory adaptation, whereas the conditional ablation of the gene has more severe effect because cells have no time to adapt.

AurkA and spindle orientation

In an epithelial tissue, well-established apical-basolateral polarity is necessary in order for the cell divisions to be oriented correctly. Therefore, perturbations in apical-basolateral polarity are likely to lead to misorientation in spindle orientation. Our analysis of the spindle orientation in the embryo epidermis, show that in the AurkA heterozygous epidermis at E14.5 there was an increase of perpendicular cell divisions compared to controls, while E15.5 AurkA heterozygous embryo epidermis presented a decrease in the number of perpendicular cell divisions compared to controls. Of note, additional polarity proteins have been identified in the regulation of oriented cell divisions in the epidermis. These include the scaffolding proteins NuMA and Inscutable which localize to the suprabasal differentiating cell during asymmetric cell division. Our results resemble the model overexpressing the protein Inscutable in skin, where, at first an increase in asymmetric cell divisions was observed, but the effect is later reversed upon prolonged Inscutable expression, probably to achieve an equilibrium between proliferation and differentiation (Poulson and Lechler, 2010). This phenotype is accompanied by

disassociation of the protein NuMA from the apical surface. Possibly, AurkA is also required for the proper localization of the spindle orientation determinants. Further studies will probably shed light about the potential roles for AurkA in the regulation of oriented cell divisions via these mitotic polarity proteins in the skin.

AurkA and cell polarity concepts extending beyond

Many polarity protein complexes, including the Par complex, are conserved across different types of cell polarity. In order to see whether the polarity role of AurkA also extends further than apical-basolateral polarity in the skin epidermis, we analyzed front-rear polarity in keratinocytes. Indeed, our results confirmed that AurkA plays a role in front-rear polarity, which presents a range of new possibilities for investigating how AurkA could be involved in this particular type of polarity. Could it control the cytoskeleton?

We also wanted to tests whether the polarity role of AurkA is true in other types of epithelial tissues. We used MDCK cells, an example of simple epithelia, where we also found loss of polarity upon KD or inhibition of AurkA. These findings present a unique opportunity to explore the role of AurkA in polarity, exploiting MDCK cells, as a bona-fide model of apical-basolateral polarity. This model would allow for a detailed, *in vitro* analysis of the polarity pathway in which AurkA may be involved. For example, spindle orientation during cyst formation would be interesting aspect analyze.

2. AurkA, apical-basolateral polarity, and cancer

Although prevention, diagnostics, and treatment of cancer have improved greatly in the last decades, the battle against cancer remains, and there is a long way to go before we can understand the complexity of this disease. Before understanding the malfunction of a tissue, it is necessary to understand it's homeostatic process. For this reason, it was interesting to find that loss of AurkA in skin epidermis can lead to an increase in proliferation, that was accompanied by alterations in cell polarity.

A wave of transitory proliferation in the AurkA heterozygous embryo epidermis

Possibly one of the most intriguing questions that emerged from our studies was the presence of hyperplastic patches across the AurkA heterozygous mutant epidermis. Deciphering the mechanism behind the hyperproliferative phenotype would be challenging because *in vitro* AurkA heterozygous keratinocytes proliferate at the same rate as control keratinocytes (data not shown). One possibility is that the hyperplastic phenotype is the result of responding to several cues arising from the whole tissue macroenviroment, and not a cell-autonomous proliferative response. To this regard, it is known that loss of ECAD leads to increased Notch signaling and cell survival *in vivo* (Ferreira et. al, 2011). In our *in vivo* analysis of ECAD, the diffused signal was not restricted only to the hyperplastic areas of the heterozygous epidermis, suggesting that the loss of functional AJs indeed precedes hyperproliferation. In order to understand how the loss or AurkA and cell polarity can lead to hyperproliferation in the context of a tissue, it could be interesting to explore the role of Notch in our system.

Interestingly, the epidermal hyperplastic phenotype became hypoplastic as embryos progressed through development (E16.5-E18.5). This resembles some previously published studies showing that in mammalian epidermis Myc can play both an oncogenic role, as well as stimulate differentiation (Watt et al., 2008). Activation of Myc leads to stem cell proliferation, while sustained levels of Myc activation stimulates the proliferation of stem cells to enter to trans-amplifying compartment and thereby initiate terminal differentiation. In other words, sustained levels of a proliferative cue can be converted into a differentiation cue. Perhaps, the phenotype we observed with AurkA heterozygous embryo epidermis may imply a similar mechanism. Moreover, like in the Myc-overexpression model, AurkA heterozygous, hyperplastic epidermis also exhibited a slight expansion of the spinous layer, suggesting that proliferating cells were indeed induced to differentiate.

Another intriguing observation we made was that the hyperproliferative patches found in AurkA heterozygous embryo epidermis very often had columnar structure, with a line of cells expanding upwards. Previous studies, using linear tracing experiments have shown that the proliferating keratinocytes are confined to distinct columnar units representing

epidermal proliferative units (Ghazizadeh and Taichman, 2001). Thus, our observation may imply that the partial loss of AurkA hyperactivates the proliferation of stem cells in the skin epidermis.

All the AurkA homozygous embryos presented hypoplasia and decreased proliferation. This is probably due to the cell arrest induced by the loss of both alleles of AurkA. Thus, it is reasonable to speculate that partial loss of AurkA is not sufficient to cause cell-arrest, but is sufficient to cause hyperplasia, again suggesting that AurkA plays a role other than the mitotic cycle and centrosomes regulation during division.

AurkA inhibition as a cancer treatment

Aurora kinase is commonly overexpressed in multiple tumor types. Several small-molecule inhibitors have been designed to block its function. Indeed, the AurkA inhibitor MLN8237 used in this study is currently in Phase 2 clinical trials for the treatment of stage III and IV of Melanoma. The rationale behind this treatment is to induce cell growth arrest, and thus combat the proliferating cancer cells. Indeed, the genetic depletion of AurkA in skin, in mice that develop tumors upon DMBA/TPA protocol, confirms the efficiency of reducing total AurkA skin levels, for treatment of tumors(Pérez de Castro et al., 2013). However, we believe that our findings highlight the risks of not achieving a complete reduction of AurkA activity, since this may have opposite effects. Our *in vitro* and *in vivo* data shows that loss of AurkA leads to reductions in cell polarity and cell junction stability. The consequences of the loss of ECAD in promoting tumorigenesis and epithelial to mesenchymal transition events in malignant tumors are well characterized (Faraldo et al., 1997). Moreover, it has been recently shown that loss of Par3 leads to the development of carcinomas in both epidermis and mammary epithelia (Iden et al., 2012; McCaffrey et al., 2012). Although preliminary, our results employing the DMBA/TPA carcinogenesis protocol in the AurkA heterozygous skin of adult mice revealed a reduction in the latency of appearance of papillomas compared to controls. Although this can be a combination of proliferative properties combined with the acquisition of genomic instability, our results suggests that further understanding is needed to develop effective therapies against AurkA, to avoid any possible adverse effects in the responsiveness of tumors.

In closing, by partially reducing the levels of AurkA in the epidermis, we have uncovered a novel role for this protein, underlying an hyperproliferative transitory response during skin development in a process associated with loss of apical-basal polarity. These findings now open new avenues for exploring the relationship between AurkA, loss of intercellular adhesion molecules and cell polarity, hallmarks of several human skin disorders and disease.

Conclusions

Objective 1: Characterize the epidermis of the K14CreERT2; AurkA$^{\Delta cp/+}$ and K14CreERT2; AurkA$^{\Delta cp/\Delta cp}$ mice during skin development.

a. In our system of conditionally induced AurkA ablation in the epidermis, Cre gene begins to be expressed at E14.5, and AurkA protein levels are significantly reduced in both heterozygous and homozygous embryos, confirming that the system works, and defining the kinetic of the genetic recombination event.

b. Heterozygous loss of AurkA in the developing epidermis does not result in any apparent macroscopical defects, compared to the embryos with homozygous loss of AurkA in skin. However, it leads to a transitory hyperplastic phenotype in the epidermis at E14.5-E16.5, characterized by increased proliferation and expansion of differentiated layers.

Objective 2: Investigate the role of AurkA in the regulation of apical- basolateral polarity in epidermal cells, both *in vivo* and *in vitro*

a. pAurkA is localized at the cell cortex in both basal and differentiated layers of the embryo epidermis, as well as the cell cortex of differentiated keratinocytes *in vitro*
b. Loss of AurkA leads to aberrations in the localization of the Par complex proteins Par3 and aPKC *in vivo*, while *in vitro,* deficiency or inhibition of AurkA leads to downregulation of the two proteins. Inhibition of aPKC does not affect the localization of pAurkA at the cortex, but does lead to a delay in adherens junction formation.
c. Loss of AurkA leads to reductions in adherens junctions *in vivo*, consistent with the observations made *in vitro,* in which the deficiency or inhibition of AurkA also leads to reductions of ECAD protein levels. Polarity and adhesion events are mutually modulated, since the loss of cadherins, *in vitro*, leads to the loss of the cortical localization of pAurkA.
d. Deficiency or inhibition of AurkA leads to a delay in tight junction formation *in vitro*, but it does not affect the total levels of the tight junction protein Occludin.

e. Deficiency of AurkA in the embryo epidermis leads to misalignments of oriented cell divisions.
f. Loss of AurkA leads to loss of cell front-rear polarity during cell migration.
g. Deficiency or inhibition of AurkA in the MDCK cell system of cell polarity, also leads to the loss of the cortical localization of aPKC and Par3, as well loss of polarized cyst-formation in Matrigel, suggesting a fundamental role for AurkA in the maintenance of apical-basolateral polarity in epithelia.
h. Preliminary results indicate that the heterozygous loss of AurkA, reduces the latency of papilloma appearance in the skin using the DMBA/TPA carcinogenesis model. This maybe due to a combination of the acquisition of genomic instability, loss of cell polarity and increased proliferation.

Conclusiones

Objetivo 1: Caracterizar la epidermis de ratones K14CreERT2; AurkA$^{\Delta ep/+}$ y K14CreERT2; AurkA$^{\Delta ep/\Delta ep}$ durante el desarrollo embrionario.

a. En nuestros modelos de ratón modificados genéticamente para eliminar/reducir los niveles de AurkA en la epidermis, el gen que codifica la enzima Cre comienza a expresarse en el día 14.5 del desarrollo embrionario (E14.5), y los niveles de AurKA se reducen significativamente tanto en ratones homocigotos como heterocigotos, confirmando que el sistema funciona.
b. Los embriones AurkA heterocigotos y homocigotos presentan un fenotipo visible macroscópicamente el día E17.5. A nivel microscópico, en el estadío E14.5-E15.5 ya se detectan áreas de hiperplasia en la epidermis. Los embriones heterocigotos que presentan dicha hiperplasia muestran además un aumento en la proliferación y una expansión de las capas diferenciadas de la epidermis.

Objetivo 2: Investigar el papel de AurkA en la regulación de la formación y el mantenimiento de la polaridad apico-basal en células de la epidermis *in vivo* e *in vitro*.

a. pAurkA se localiza en el cortex de las células de la epidermis embrionaria, tanto de las capas basales como de las diferenciadas. *In vitro*, pAurkA se localiza en el cortex de los queratinocitos diferenciados.
b. La pérdida de AurkA conduce a una pérdida de la localización correcta de las proteínas Par3 y aPKC *in vivo*; mientras que *in vitro,* la ausencia o la inhibición química de AurkA inducen una disminución en los niveles de expresión de ambas proteínas. La inhibición de aPKC no afecta a la localización de pAurKA en el cortex celular, pero conduce a un retraso en la formación de las uniones adherentes.
c. La pérdida de AurkA genera alteraciones en las uniones adherentes *in vivo*; mientras que *in vitro*, la ausencia o la inhibición química de AurkA inducen una disminución en los niveles de expresión de ECAD. Por otro lado, la pérdida de las cadherinas *in vitro* induce la pérdida de la localización cortical de pAurkA.
d. La ausencia o la inhibición química de AurkA inducen un retraso en la formación de las uniones estrechas *in vitro*, pero no cambian los niveles de expresión de las proteínas de las uniones estrechas ocludina y ZO1.
e. La pérdida de AurkA en la epidermis del embrión induce alteraciones en la orientación de las divisiones celulares.

f. La pérdida de AurkA genera alteraciones en la polaridad *front-rear* durante la migración celular.
g. La ausencia o la inhibición química de AurkA en las células MDCK conducen a la pérdida de la localización cortical de aPKC y Par3, así como a la pérdida de la estructura polarizada de los cultivos 3D en matrigel.
h. Después del tratamiento con DMBA/TPA, los ratones heterocigotos para AurkA desarrollan papilomas más tempranamente que los ratones control.

References

- Adams, C. L. & Nelson, W. J. Cytomechanics of cadherin-mediated cell-cell adhesion. *Current opinion in cell biology* **10,** 572–7 (1998).
- Ahringer, J. Control of cell polarity and mitotic spindle positioning in animal cells. *Current Opinion in Cell Biology* **15,** 73–81 (2003).
- Balklava, Z., Pant, S., Fares, H. & Grant, B. D. Genome-wide analysis identifies a general requirement for polarity proteins in endocytic traffic. *Nature cell biology* **9,** 1066–73 (2007).
- Bayraktar, J., Zygmunt, D. & Carthew, R. W. Par-1 kinase establishes cell polarity and functions in Notch signaling in the Drosophila embryo. *Journal of cell science* **119,** 711–21 (2006).
- Bilder, D. Epithelial polarity and proliferation control: links from the Drosophila neoplastic tumor suppressors. *Genes Dev* **18,** 1909–1925 (2004).
- Blanpain, C. & Fuchs, E. Epidermal homeostasis: a balancing act of stem cells in the skin. *Nature reviews. Molecular cell biology* **10,** 207–17 (2009).
- Cayouette, M. & Raff, M. Asymmetric segregation of Numb: a mechanism for neural specification from Drosophila to mammals. *Nature neuroscience* **5,** 1265–9 (2002).
- Chen, J. & Zhang, M. The Par3/Par6/aPKC complex and epithelial cell polarity. *Experimental cell research* **319,** 1357–64 (2013).
- Chen, X. & Macara, I. G. Par-3 controls tight junction assembly through the Rac exchange factor Tiam1. *Nature cell biology* **7,** 262–9 (2005).
- Cheng, N. N., Kirby, C. M. & Kemphues, K. J. Control of Cleavage Spindle Orientation in. *Genetics* (1995).
- Cicalese, A. *et al.* The tumor suppressor p53 regulates polarity of self-renewing divisions in mammary stem cells. *Cell* **138,** 1083–95 (2009).
- Cowley, D. O. *et al.* Aurora-A kinase is essential for bipolar spindle formation and early development. *Molecular and cellular biology* **29,** 1059–71 (2009).
- David, D. J. V, Tishkina, A. & Harris, T. J. C. The PAR complex regulates pulsed actomyosin contractions during amnioserosa apical constriction in Drosophila. *Development (Cambridge, England)* **137,** 1645–55 (2010).
- Denning, M. F. Tightening the epidermal barrier with atypical PKCs. *The Journal of investigative dermatology* **127,** 742–4 (2007).

- Dupin, I., Camand, E. & Etienne-Manneville, S. Classical cadherins control nucleus and centrosome position and cell polarity. *The Journal of cell biology* **185,** 779–86 (2009).
- Etemad-Moghadam, B., Guo, S. & Kemphues, K. J. Asymmetrically distributed PAR-3 protein contributes to cell polarity and spindle alignment in early C. elegans embryos. *Cell* **83,** 743–52 (1995).
- Etienne-Manneville, S. & Hall, a. Integrin-mediated activation of Cdc42 controls cell polarity in migrating astrocytes through PKCzeta. *Cell* **106,** 489–98 (2001).
- Etienne-Manneville, S. Cdc42--the centre of polarity. *Journal of cell science* **117,** 1291–300 (2004).
- Famulski, J. K. & Solecki, D. J. New spin on an old transition: epithelial parallels in neuronal adhesion control. *Trends in neurosciences* **36,** 163–73 (2013).
- Faraldo, M. L., Rodrigo, I., Behrens, J., Birchmeier, W. & Cano, a. Analysis of the E-cadherin and P-cadherin promoters in murine keratinocyte cell lines from different stages of mouse skin carcinogenesis. *Molecular carcinogenesis* **20,** 33–47 (1997).
- Fu, J., Bian, M., Jiang, Q. & Zhang, C. Roles of Aurora kinases in mitosis and tumorigenesis. *Molecular cancer research : MCR* **5,** 1–10 (2007).
- Fuchs, E. Scratching the surface of skin development. *Nature* **445,** 834–42 (2007).
- Fuchs, E. & Horsley, V. More than one way to skin . . . *Genes & development* **22,** 976–85 (2008).
- Garrard, S. M. *et al.* Structure of Cdc42 in a complex with the GTPase-binding domain of the cell polarity protein, Par6. *The EMBO journal* **22,** 1125–33 (2003).
- Gautschi, O. *et al.* Aurora kinases as anticancer drug targets. *Clinical cancer research : an official journal of the American Association for Cancer Research* **14,** 1639–48 (2008).
- Ghazizadeh, S. & Taichman, L. B. Multiple classes of stem cells in cutaneous epithelium: a lineage analysis of adult mouse skin. *The EMBO journal* **20,** 1215–22 (2001).

- Gibson, M. C. & Perrimon, N. Apicobasal polarization: epithelial form and function. *Current Opinion in Cell Biology* **15,** 747–752 (2003).
- Gotta, M., Abraham, M. C. & Ahringer, J. CDC-42 controls early cell polarity and spindle orientation in C. elegans. *Current biology : CB* **11,** 482–8 (2001).
- Guillot, C. & Lecuit, T. Mechanics of epithelial tissue homeostasis and morphogenesis. *Science (New York, N.Y.)* **340,** 1185–9 (2013).
- Gurumurthy, S. *et al.* LKB1 deficiency sensitizes mice to carcinogen-induced tumorigenesis. *Cancer research* **68,** 55–63 (2008).
- Hao, Y. *et al.* Par3 controls epithelial spindle orientation by aPKC-mediated phosphorylation of apical Pins. *Current Biology* **20,** 1809–1818 (2010).
- Hardman, M. J., Sisi, P., Banbury, D. N. & Byrne, C. Patterned acquisition of skin barrier function during development. *Development (Cambridge, England)* **125,** 1541–52 (1998).
- Harris, T. J. C. & Peifer, M. Adherens junction-dependent and -independent steps in the establishment of epithelial cell polarity in Drosophila. *The Journal of cell biology* **167,** 135–47 (2004).
- Huang, R. Y.-J., Guilford, P. & Thiery, J. P. Early events in cell adhesion and polarity during epithelial-mesenchymal transition. *Journal of cell science* **125,** 4417–22 (2012).
- Humbert, P., Russell, S. & Richardson, H. Dlg, Scribble and Lgl in cell polarity, cell proliferation and cancer. *BioEssays : news and reviews in molecular, cellular and developmental biology* **25,** 542–53 (2003).
- Hutterer, A. *et al.* Mitotic activation of the kinase Aurora-A requires its binding partner Bora. *Developmental cell* **11,** 147–57 (2006).
- Iden, S. *et al.* Tumor type-dependent function of the par3 polarity protein in skin tumorigenesis. *Cancer cell* **22,** 389–403 (2012).
- Itoh, M., Nagafuchi, a, Moroi, S. & Tsukita, S. Involvement of ZO-1 in cadherin-based cell adhesion through its direct binding to alpha catenin and actin filaments. *The Journal of cell biology* **138,** 181–92 (1997).
- Izumi, Y. *et al.* An Atypical PKC Directly Associates and Colocalizes at the Epithelial Tight Junction with ASIP, a Mammalian Homologue of. *The Journal of cell biology* **143,** 95–106 (1998).

- Jansen, M., Paul, J., Klooster, T. E. N., Offerhaus, G. J. & Clevers, H. LKB1 and AMPK Family Signaling : The Intimate Link Between Cell Polarity and Energy Metabolism. *Physiology Review* 777–798 (2009). doi:10.1152/physrev.00026.2008.
- Januschke, J. & Gonzalez, C. Drosophila asymmetric division, polarity and cancer. *Oncogene* **27,** 6994–7002 (2008).
- Joberty, G., Petersen, C., Gao, L. & Macara, I. G. The cell-polarity protein Par6 links Par3 and atypical protein kinase C to Cdc42. *Nature cell biology* **2,** 531–9 (2000).
- Kemphues, K. J., Priess, J. R., Morton, D. G. & Cheng, N. S. Identification of genes required for cytoplasmic localization in early C. elegans embryos. *Cell* **52,** 311–20 (1988).
- Khazaei, M. R. & Püschel, A. W. Phosphorylation of the par polarity complex protein Par3 at serine 962 is mediated by aurora a and regulates its function in neuronal polarity. *The Journal of biological chemistry* **284,** 33571–9 (2009).
- Kirschner, M., Gerhart, J. & Mitchison, T. Molecular " Vitalism " Review University of California at Berkeley. *Cell* **100,** 79–88 (2000).
- Kirschner, N. *et al.* CD44 regulates tight-junction assembly and barrier function. *The Journal of investigative dermatology* **131,** 932–43 (2011).
- Klezovitch, O., Fernandez, T. E., Tapscott, S. J. & Vasioukhin, V. Loss of cell polarity causes severe brain dysplasia in Lgl1 knockout mice. *Genes & Development* **18,** 559–571 (2004).
- Knoblich, J. a. Mechanisms of asymmetric stem cell division. *Cell* **132,** 583–97 (2008).
- Kollareddy, M., Dzubak, P., Zheleva, D. & Hajduch, M. Aurora kinases: structure, functions and their association with cancer. *Biomedical papers of the Medical Faculty of the University Palacký, Olomouc, Czechoslovakia* **152,** 27–33 (2008).
- Laprise, P. & Tepass, U. Novel insights into epithelial polarity proteins in Drosophila. *Trends in cell biology* **21,** 401–8 (2011).
- Lechler, T. & Fuchs, E. Asymmetric cell divisions promote stratification and differentiation of mammalian skin. *Nature* **437,** 275–80 (2005).

- Lorenzo, C., Liao, Q., Hardwicke, M. A. & Ducommun, B. Pharmacological inhibition of aurora-A but not aurora-B impairs interphase microtubule dynamics. *Cell cycle (Georgetown, Tex.)* **8,** 1733–7 (2009).
- Lu, L.-Y. *et al.* Aurora A is essential for early embryonic development and tumor suppression. *The Journal of biological chemistry* **283,** 31785–90 (2008).
- Lu, L.-Y. *et al.* Aurora A is essential for early embryonic development and tumor suppression. *The Journal of biological chemistry* **283,** 31785–90 (2008).
- Macara, I. G. Parsing the polarity code. *Nature reviews. Molecular cell biology* **5,** 220–31 (2004).
- Macara, I. G. & Mili, S. Polarity and differential inheritance--universal attributes of life? *Cell* **135,** 801–12 (2008).
- Manuscript, A. NIH Public Access. **133,** 78–86 (2013).
- Manuscript, A. Building Epithelial Tissues from Skin Stem Cells. **14,** 333–350 (2009).
- Manuscript, A. NIH Public Access. *Nat Rev Mol Cell Biol* **12,** 565–580 (2012).
- Manuscript, A. & Epidermal, R. NIH Public Access. *Developmental cell* **19,** 727–739 (2011).
- Martin-Belmonte, F. & Perez-Moreno, M. Epithelial cell polarity, stem cells and cancer. *Nature Reviews Cancer* **12,** 23–38 (2012).
- McCaffrey, L. M. & Macara, I. G. The Par3/aPKC interaction is essential for end bud remodeling and progenitor differentiation during mammary gland morphogenesis. *Genes & development* **23,** 1450–60 (2009).
- McCaffrey, L. M. & Macara, I. G. Epithelial organization, cell polarity and tumorigenesis. *Trends in cell biology* **21,** 727–35 (2011).
- McCaffrey, L. M., Montalbano, J., Mihai, C. & Macara, I. G. Loss of the Par3 polarity protein promotes breast tumorigenesis and metastasis. *Cancer cell* **22,** 601–14 (2012).
- Mège, R.-M., Gavard, J. & Lambert, M. Regulation of cell-cell junctions by the cytoskeleton. *Current opinion in cell biology* **18,** 541–8 (2006).

- Meng, W. & Takeichi, M. Adherens Junction : Molecular. *CSH Perspectives in Biology* 1–13 (2009).
- Meşe, G., Richard, G. & White, T. W. Gap junctions: basic structure and function. *The Journal of investigative dermatology* **127,** 2516–24 (2007).
- Miller, C. J. & Davidson, L. a. The interplay between cell signalling and mechanics in developmental processes. *Nature reviews. Genetics* **14,** 733–44 (2013).
- Mogilner, A., Allard, J. & Wollman, R. Cell polarity: quantitative modeling as a tool in cell biology. *Science (New York, N.Y.)* **336,** 175–9 (2012).
- Mori, D. *et al.* An essential role of the aPKC-Aurora A-NDEL1 pathway in neurite elongation by modulation of microtubule dynamics. *Nature cell biology* **11,** 1057–68 (2009).
- Muroyama, A. & Lechler, T. Polarity and stratification of the epidermis. *Seminars in cell & developmental biology* **23,** 890–6 (2012).
- Murray, N. R., Kalari, K. R. & Fields, A. P. Mechanisms in Cancer. *Journl of cell physiology* **226,** 879–887 (2012).
- Muthuswamy, S. K. & Xue, B. Cell polarity as a regulator of cancer cell behavior plasticity. *Annual review of cell and developmental biology* **28,** 599–625 (2012).
- Muthuswamy, S. K. & Xue, B. Cell polarity as a regulator of cancer cell behavior plasticity. *Annual review of cell and developmental biology* **28,** 599–625 (2012).
- Nagai-Tamai, Y., Mizuno, K., Hirose, T., Suzuki, A. & Ohno, S. Regulated protein-protein interaction between aPKC and PAR-3 plays an essential role in the polarization of epithelial cells. *Genes to cells : devoted to molecular & cellular mechanisms* **7,** 1161–71 (2002).
- Niessen, C. M. Tight junctions/adherens junctions: basic structure and function. *The Journal of investigative dermatology* **127,** 2525–32 (2007).
- Niessen, M. T., Iden, S. & Niessen, C. M. The in vivo function of mammalian cell and tissue polarity regulators--how to shape and maintain the epidermal barrier. *Journal of cell science* **125,** 3501–10 (2012).

- Nikonova, A. S., Astsaturov, I., Serebriiskii, I. G., Dunbrack, R. L. & Golemis, E. a. Aurora A kinase (AURKA) in normal and pathological cell division. *Cellular and molecular life sciences : CMLS* **70,** 661–87 (2013).
- Nola, S. *et al.* Ajuba is required for Rac activation and maintenance of E-cadherin adhesion. *The Journal of cell biology* **195,** 855–71 (2011).
- Ochi, T., Fujiwara, H. & Yasukawa, M. Aurora-A kinase: a novel target both for cellular immunotherapy and molecular target therapy against human leukemia. *Expert opinion on therapeutic targets* **13,** 1399–410 (2009).
- Ozdamar, B. *et al.* Regulation of the polarity protein Par6 by TGFbeta receptors controls epithelial cell plasticity. *Science (New York, N.Y.)* **307,** 1603–9 (2005).
- Peng, Y.-H., Yang, W.-K., Lin, W.-H., Lai, T.-T. & Chien, C.-T. Nak regulates Dlg basal localization in Drosophila salivary gland cells. *Biochemical and biophysical research communications* **382,** 108–13 (2009).
- Pérez de Castro, I. *et al.* Requirements for Aurora-A in tissue regeneration and tumor development in adult mammals. *Cancer research* **73,** 6804–15 (2013).
- Poulson, N. D. & Lechler, T. Robust control of mitotic spindle orientation in the developing epidermis. *The Journal of cell biology* **191,** 915–22 (2010).
- Pugacheva, E. N., Jablonski, S. A., Hartman, T. R., Henske, E. P. & Golemis, E. A. primary cilium. *Cell* **129,** 1351–1363 (2008).
- Pugacheva, E. N., Jablonski, S. a, Hartman, T. R., Henske, E. P. & Golemis, E. a. HEF1-dependent Aurora A activation induces disassembly of the primary cilium. *Cell* **129,** 1351–63 (2007).
- Quyn, A. J. *et al.* Spindle orientation bias in gut epithelial stem cell compartments is lost in precancerous tissue. *Cell stem cell* **6,** 175–81 (2010).
- Regan, J. L. *et al.* Aurora A kinase regulates mammary epithelial cell fate by determining mitotic spindle orientation in a Notch-dependent manner. *Cell reports* **4,** 110–23 (2013).
- Royer, C. & Lu, X. Epithelial cell polarity: a major gatekeeper against cancer? *Cell death and differentiation* **18,** 1470–7 (2011).
- Sardon, T. *et al.* Uncovering new substrates for Aurora A kinase. *EMBO reports* **11,** 977–84 (2010).

- Siller, K. H. & Doe, C. Q. Spindle orientation during asymmetric cell division. *Nature cell biology* **11,** 365–74 (2009).
- Tanos, B. & Rodriguez-Boulan, E. The epithelial polarity program: machineries involved and their hijacking by cancer. *Oncogene* **27,** 6939–57 (2008).
- Tepass, U. The apical polarity protein network in Drosophila epithelial cells: regulation of polarity, junctions, morphogenesis, cell growth, and survival. *Annual review of cell and developmental biology* **28,** 655–85 (2012).
- Thompson, B. J. Cell polarity: models and mechanisms from yeast, worms and flies. *Development (Cambridge, England)* **140,** 13–21 (2013).
- Thompson, B. J., Pichaud, F. & Röper, K. Sticking together the Crumbs - an unexpected function for an old friend. *Nature reviews. Molecular cell biology* **14,** 307–14 (2013).
- Tinkle, C. L., Pasolli, H. A., Stokes, N. & Fuchs, E. New insights into cadherin function in epidermal sheet formation and maintenance of tissue integrity. *Proceedings of the National Academy of Sciences of the United States of America* **105,** 15405–10 (2008).
- Torchia, E. C., Zhang, L., Huebner, A. J., Sen, S. & Roop, D. R. Aurora kinase-A deficiency during skin development impairs cell division and stratification. *The Journal of investigative dermatology* **133,** 78–86 (2013).
- Tunggal, J. a *et al.* E-cadherin is essential for in vivo epidermal barrier function by regulating tight junctions. *The EMBO journal* **24,** 1146–56 (2005).
- Umeda, K. *et al.* ZO-1 and ZO-2 independently determine where claudins are polymerized in tight-junction strand formation. *Cell* **126,** 741–54 (2006).
- Vader, G. & Lens, S. M. a. The Aurora kinase family in cell division and cancer. *Biochimica et biophysica acta* **1786,** 60–72 (2008).
- Vasioukhin, V., Bauer, C., Yin, M. & Fuchs, E. Directed actin polymerization is the driving force for epithelial cell-cell adhesion. *Cell* **100,** 209–19 (2000).
- Vasioukhin, V. & Fuchs, E. Actin dynamics and cell-cell adhesion in epithelia. *Current opinion in cell biology* **13,** 76–84 (2001).

- Wang, X. *et al.* Overexpression of aurora kinase A in mouse mammary epithelium induces genetic instability preceding mammary tumor formation. *Oncogene* **25,** 7148–58 (2006).
- Wang, Y.-C., Khan, Z., Kaschube, M. & Wieschaus, E. F. Differential positioning of adherens junctions is associated with initiation of epithelial folding. *Nature* **484,** 390–3 (2012).
- Watt, F. M., Frye, M. & Benitah, S. A. MYC in mammalian epidermis: how can an oncogene stimulate differentiation? *Nature reviews. Cancer* **8,** 234–42 (2008).
- Williams, S. E., Beronja, S., Pasolli, H. A. & Fuchs, E. Asymmetric cell divisions promote Notch-dependent epidermal differentiation. *Nature* **470,** 353–8 (2011).
- Wirtz-Peitz, F., Nishimura, T. & Knoblich, J. a. Linking cell cycle to asymmetric division: Aurora-A phosphorylates the Par complex to regulate Numb localization. *Cell* **135,** 161–73 (2008).
- Wu, C.-C. *et al.* p53 negatively regulates Aurora A via both transcriptional and posttranslational regulation. *Cell cycle (Georgetown, Tex.)* **11,** 3433–42 (2012).
- Yamada, M., Hirotsune, S. & Wynshaw-Boris, A. The essential role of LIS1, NDEL1 and Aurora-A in polarity formation and microtubule organization during neurogensis. *Cell adhesion & migration* **4,** 180–4 (2010).
- Zallen, J. a. Planar polarity and tissue morphogenesis. *Cell* **129,** 1051–63 (2007).